

SPECIFICATION

METHOD FOR IMPROVING FERTILITY OF HYBRID PLANTS COMPRISING
PLACING FERTILITY RESTORER GENES INTO MULTIPLE GENE LOCI

TECHNICAL FIELD

5 [0001] The present application claims priority based on Japanese Patent Application No. 2003-173927 filed on June 18, 2003.

[0002] Technical Field of the Invention

The present invention relates to hybrid plants in which a plurality of fertility restorer genes have been introduced at multiple gene loci, and to the use thereof.

[0003] Background Art

When two varieties of a self-fertilizing plant such as rice are to be crossed, it is necessary to first avoid self-fertilization by removing all stamens in a glumous flower just before it flowers. The flower is then fertilized using pollen from the pollen parent variety with which it is being crossed. However, a crossing technique that involves such manual operations is poorly suited for the production of a large quantity of hybrid seed on a commercial scale.

[0004] Accordingly, hybrid cultivars are produced by a three-line method which makes use of cytoplasmic male sterility. As used herein, "a three-line method" refers to a procedure that employs a sterile line containing male-sterile cytoplasm, a restorer line having a gametophytic fertility restorer gene, and a maintainer line having the same nuclear genes as the sterile line but

lacking sterile cytoplasm. Using these three lines, (i) hybrid seeds can be obtained by fertilizing the sterile line with pollen from the restorer line, and (ii) the sterile line can be maintained by fertilizing it with
5 pollen from the maintainer line.

[0005] The male-sterile cytoplasm and fertility restorer genes encoded in the nucleus are employed to commercially produce hybrid seed. Fertility restorer genes are classified as gametic or sporophytic type
10 according to their mechanism of action. In the case of gametic fertility restorer genes, the genotype of the pollen determines whether or not the pollen fertility is restored; known examples include the fertility restorer gene Rf-1 for rice BT-type male-sterile cytoplasm and the
15 restorer gene for maize S-type male-sterile cytoplasm. In the case of sporophytic fertility restorer genes, the genotype of the plant that produces the pollen determines whether or not the pollen fertility is restored; known examples include the fertility restorer gene for rice
20 WA-type male-sterile cytoplasm and the fertility restorer gene for maize T-type male-sterile cytoplasm.

[0006] When a hybrid is bred by using a gametic fertility restorer gene, the anther of the hybrid variety shows a 1:1 segregation of pollen carrying the fertility
25 restorer gene and pollen lacking the gene, and so the theoretical pollen fertility is 50%. This is half of the theoretical pollen fertility of 100% for a common variety, and has been of concern as a factor that lowers the

stability of seed production in hybrids. In fact, hybrids obtained using rice BT-type male-sterile cytoplasm and the fertility restorer gene Rf-1 are generally known to have a poor cold hardiness, which is thought to be attributable
5 to the low (50%) theoretical pollen fertility.

[0007] The following problems are associated with sporophytic fertility restorer genes. Although fertility restoration in rice WA cytoplasm is thought to be imparted by a plurality of fertility restorer genes, the number of
10 such genes and their chromosomal positions have not been identified in detail. Hence, to be used in cross breeding, a restorer line for WA cytoplasm, in addition to having excellent properties such as yield and plant type, must also have the ability, as demonstrated in a seed fertility
15 study on F₁ plants obtained after being crossed with a sterile line, to completely restore fertility to WA cytoplasm. Regardless of the excellence of properties other than the fertility restoring ability, if the seed fertility in F₁ plants obtained after the restorer line
20 has been crossed with a WA cytoplasmic male-sterile line is incomplete, use as a restorer line will be impossible. Moreover, as noted above, because the number and positions of fertility restorer genes in restorer lines have not yet been precisely identified, it is difficult to improve only
25 the fertility restoring ability while retaining the other properties.

[0008] A desire thus exists for a method of preparing hybrid cultivars having a high fertility.

- Patent Publication No.1: Japanese Patent Public Disclosure No. 2002-345485
- Patent Publication No.2: WO 02/014506 A1
- Patent Publication No.3: WO 03/027290 A1
- 5 Patent Publication No.4: WO 02/019803 A1
- Non-Patent Publication No.1:
- Ahmed, M.I., and Siddiq, E.A. (1998). Rice. In Hybrid cultivar development, S.S. Banga and S.K. Banga, eds (Berlin: Springer Verlag), pp. 221-256.
- 10 Non-Patent Publication No.2:
- Dhillon, B.S. (1998). Maize. In Hybrid cultivar development, S.S. Banga and S.K. Banga, eds (Berlin: Springer Verlag, pp. 282-315.
- Non-Patent Publication No.3:
- 15 Wen, L.&Chase, C.D.(1999). Curr.Genet.35,p.521-526
- Non-Patent Publication No.4:
- Fukuta et al. 1992, Jpn J. Breed. 42 (supl.1) p.164-165
- Non-Patent Publication No.5:
- 20 Hiei et al., Plant Journal(1994),6(2),p.272-282
- Non-Patent Publication No.6:
- Komari et al., Plant Journal(1996) 10, p.165-174
- Non-Patent Publication No.7:
- Ditta et al., Proc.Natl.Acad.Sci. USA
- 25 (1980),77:p.7347-7351
- Non-Patent Publication No.8:
- Lemas et al., Plasmid 1992,27,p.161-163
- Non-Patent Publication No.9:

Cui, X., Wise, R.P. and Schanble, P.S. (1996) The rf2 nuclear restorer gene of male-sterile T-cytoplasm maize. *Science*, 272, 1334-1336

Non-Patent Publication No.10:

5 Liu, F., Cui, X., Horner, H.T., Weiner, H. and Schnable, P.S. (2001) Mitochondrial aldehyde dehydrogenase activity is required for male fertility in maize. *The Plant Cell*, 13, 1063-1078

Non-Patent Publication No.11:

10 Michaels and Amasino 1998, *The Plant Journal* 14(3) p.381-385

Non-Patent Publication No.12:

Neff et al. 1998, *The plant Journal* 14(3) p.387-392

Non-Patent Publication No.13:

15 Komari, T., Saito, Y., Nakakido, F., and Kumashiro, T. (1989). Efficient selection of somatic hybrids in *Nicotiana tabacum* L. using a combination of drug-resistance markers introduced by transformation. *Theor. Appl. Genet.* 77, 547-552.

20 Non-Patent Publication No.14:

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., and Lipman, D.J. (1990). Basic local alignment search tool. *J. Mol. Biol.* 215, 403-410.

Non-Patent Publication No.15:

25 Komori, T., Yamamoto, T., Takemori, N., Kashihara, M., Matsushima, H., and Nitta, N. (2002). Fine mapping of a restorer gene, Rf-1, that restores the BT-type cytoplasmic male sterility. *Breed. Res.* 4 (Suppl.2), 243.

Non-Patent Publication No.16:

Harushima, Y., et al. (1998). A high-density rice genetic linkage map with 2275 markers using a single F2 population. *Genetics* 148, 479-494.

5 Non-Patent Publication No.17:

Kariya, K. (1989). Sterility caused by cooling treatment at the flowering stage in rice plants III. Establishment of a method of in vitro pollen germination. *Jap. J. Crop Sci.* 58, 96-102.

10 DISCLOSURE OF THE INVENTION

PROBLEMS TO BE SOLVED BY THE INVENTION

[0009] An object of the present invention is to provide a hybrid plant having a high fertility. The hybrid plants of the invention are characterized by having 15 two or more copies of a fertility restorer gene at two or more gene loci which do not have a complete linkage relationship.

[0010] In the present invention, the phrase "gene loci which do not have a complete linkage relationship" 20 preferably refers to gene loci on distinct chromosomes.

[0011] In this invention, the fertility restorer gene is preferably a gametic fertility restorer gene, and more preferably the rice restorer gene for BT-type male sterility.

25 [0012] Another object of the invention is to provide a method for producing hybrid plants, which method comprises introducing a fertility restorer gene by genetic engineering and placing two or more copies of a fertility

restorer gene at two or more gene loci which do not have a complete linkage relationship.

MEANS FOR SOLVING THE PROBLEMS

[0013] As a result of extensive investigations, the 5 present inventors have succeeded in obtaining a hybrid plant of a high fertility and have ultimately arrived at the present invention.

[0014] Hybrid Plant

Accordingly, the present invention provides a hybrid 10 plant of a high fertility. The hybrid plant of the invention is characterized by having two or more copies of a fertility restorer gene at two or more gene loci which do not have a complete linkage relationship.

[0015] In the plant, during the formation of pollen as 15 the gametophytes, meiosis occurs and the respective pairs of homologous chromosomes segregate. Hence, when a hybrid cultivar is bred using a gametic fertility restorer gene and male-sterile cytoplasm, the hybrid anther exhibits a 1:1 segregation of pollen carrying the fertility restorer 20 gene and pollen lacking the gene, resulting in a theoretical pollen fertility of 50%. The hybrid plant of the invention is characterized in that a) two or more copies of the fertility restorer gene are present, and b) these copies of the gene are located at two or more gene 25 loci which do not have complete linkage relationship. It thus has the advantage that when pollen is formed by meiosis, there is a higher possibility that a gametic restorer gene will be present on one of the chromosomes.

[0016] To illustrate, in rice, which has 12 pairs of homologous chromosomes, with gene transfer and repeated crossing, a gametic restorer gene may be located on, for example, chromosomes 6, 7 and 10. When pollen is formed,

5 the homologous chromosomes which contain the gametic restorer gene and the homologous chromosomes which do not contain this gene segregate independently of the segregation with the other pair of homologous chromosomes. As a result, pollen which carries the gametic fertility

10 restorer gene in three places (chromosomes 6, 7 and 10), pollen which carries it in two places (chromosomes 6 and 7, chromosomes 6 and 10, or chromosomes 7 and 10), pollen which has it in one place (chromosome 6, 7 or 10), and pollen which has it in 0 places forms in a ratio of

15 1:3:3:1. The present inventors have shown that gametic fertility restorer genes introduced by genetic engineering function in the same way as intrinsic genes, that fertility can be obtained even if the pollen has a single gametic fertility restorer gene, and that pollen having a

20 multiple gametic fertility restorer genes develops normally. Therefore, theoretically all of the pollen aside from the 1/8 fraction which has no gametic fertility restorer gene whatsoever, that is, 87.5% of the pollen, has fertility. In Example 4 described later in the

25 specification, three-loci Rf-1 heterozygotes are shown to have a pollen fertility of about 87.5%, demonstrating that the above theory is correct.

[0017] To explain the technical features of the

invention, examples were given above in which two or more copies of a fertility restorer gene are located on distinct chromosomes. However, even if the genes are present at a multiple, for example, two loci on the same 5 chromosome, so long as there is some degree of genetic distance there between, they are inherited independently as if they were located on distinct chromosomes.

Alternatively, even if they are not inherited completely independently, so long as they do not behave in complete 10 unison, it is possible to achieve the object of this invention, which is to enhance pollen fertility by placing two or more copies of a fertility restorer gene at multiple gene loci. Hence, in this specification, "not have complete linkage relationship" includes not only 15 cases where the genes are located on distinct chromosomes and so-called "independent cases" where the genes are likewise inherited completely independently, but also "cases where the gene loci exist in a close or moderate linkage relationship" which, although not independent, is 20 not completely linked. Without being limitative, when two gene loci are separated by a distance of at least about 1 cM, and preferably at least about 5 cM, both are inherited without behaving in complete unison; that is, they can be said to be "not have a complete linkage relationship"

25 [0018] With regard to sporophytic fertility restorer genes, it is entirely conceivable that the fertility restorer gene Rf-1 for BT cytoplasm exhibits some fertility restoring ability in WA cytoplasm. Moreover,

there is a possibility that the degree of restoration will be enhanced by the placement of a plurality of copies of the Rf-1 gene. Experiments to ascertain these points are currently being carried out.

- 5 [0019] As noted above, the hybrid plant of the invention, which is a hybrid plant having two or more copies of a fertility restorer gene at two or more gene loci which do not have complete linkage relationship, has a high pollen fertility compared with fertility restorer
10 gene single-locus heterozygotes containing only one copy of the fertility restorer gene (prior-art hybrid plants). Moreover, the cold hardiness, that is, the seed fertility under a low temperature condition, is also improved (Example 7). Here, "under a low temperature condition"
15 refers to cultivation at 20 to 28°C under lighted conditions and at 15 to 23°C under dark conditions following transplantation and up to the ripening stage. For example, in the subsequently described Example 7, when cultivated for 12 hours under lighted conditions (24°C)
20 and 12 hours under dark conditions (19°C) following transplantation and up to the ripening stage, hybrid plants according to this invention (F_1 plants of FR Koshihikari crossed with 16T1-35) maintained a higher seed fertility compared to that of fertility restorer gene
25 single-locus heterozygotes (F_1 plants of MS Koshihikari crossed with FR Koshihikari), which are prior art hybrid plants having only a single copy of the fertility restorer gene.

[0020] The hybrid plants of the invention include all states: pollen, seed and adult plants.

[0021] The genus and species of the hybrid plants obtained in the invention, while not subject to any 5 specific limitation, include rice and maize. Rice is especially preferred.

[0022] The "fertility restorer gene" of the invention includes both gametic genes and sporophytic genes. In gametic genes, the genotype of the pollen determines 10 whether or not the pollen fertility is restored; known examples include the fertility restorer gene Rf-1 for rice BT-type male-sterile cytoplasm and the restorer gene for maize S-type male-sterile cytoplasm. In the case of sporophytic fertility restorer genes, the genotype of the 15 plant that produces the pollen determines whether or not the pollen fertility is restored; known examples include the fertility restorer gene for rice WA-type male-sterile cytoplasm (Ahmed and Siddiq, 1998) and the fertility restorer gene for maize T-type male-sterile cytoplasm 20 (Dhillon, 1998).

[0023] Known genes may be used as a "gametic fertility restorer gene" depending on the type of hybrid plant. For example, in the case of hybrid rice, the rice restorer gene for BT-type male sterility, Rf-1 may be used. The 25 present inventors have isolated, identified, and filed a patent application for the Rf-1 gene. In the case of hybrid maize, restorer genes for S-type male-sterile cytoplasm are known; examples include those mentioned by L.

Wen and C.D. Chase in *Curr. Genet.* 35, 521-526 (1999).

[0024] The hybrid plant of the invention includes two or more copies of a fertility restorer gene. This invention makes use of the nature of genes that are not completely linked to be inherited completely or partly independently. Therefore, even when there are a plurality of fertility restorer genes on the same chromosome, it is desirable for them to be present at a distance therebetween of at least about 1 cM, and preferably at least about 5 cM. Most preferably, it is desirable for each copy of the gene to be present on different chromosomes. Therefore, the fertility restorer gene, while not subject to any particular limitation, is present in a number that is preferably at most the number of chromosome pairs.

[0025] The hybrid plant of the invention has two or more copies of the fertility restorer gene at two or more gene loci which do not have complete linkage relationship. It is preferable for the respective copies of the genes to all exist at gene loci which do not have complete linkage relationship. However, in cases where the hybrid plant has three or more copies of the gene, if some of these genes are present at linked loci but the other genes are present at gene loci which do not have complete linkage relationship, a higher fertility can be achieved than in a single-copy (heterozygous) plant; such cases are included among the hybrid plants of the invention. For example, this includes hybrid plants containing four copies of the

gene in which two copies are present at gene loci in a linked relationship on the same chromosome and the other two copies are each present on other, separate chromosomes. The larger the number of genes that are not completely
5 linked, the higher the probability that the pollen will be fertile. Theoretically, when there is only one copy of the fertility restorer gene, this probability is 50%, but when the number of copies rises to two, three, four and five, the probability increases respectively to 75%, 87.5%,
10 93.75% and 96.875%. In Example 4 of the invention, hybrid rice having the fertility restorer gene Rf-1 at a maximum of four loci was created, and a value very close to the theoretical pollen fertility of 93.75% was observed. This showed that pollen having multiple (e.g., four) fertility
15 restorer genes also develops normally. Therefore, although not subject to particular limitation, the number of copies of the fertility restorer gene in the hybrid plant is preferably from two to the number of chromosome pairs in the host plant, and more preferably from two to
20 four.

[0026] One of the two or more copies of the fertility restorer gene in the hybrid plant of the invention may come from a fertility restorer line plant having an intrinsic fertility restorer gene. For example, rice is
25 known have an Rf-1 locus on chromosome 10 (Fukuta et al., Jpn. J. Breed. 42 (suppl. 1), 164-165 (1992)). Such an intrinsic fertility restorer gene can be used to create the hybrid plant of the invention.

[0027] Method of Producing Hybrid Plant

The present invention also provides a method for producing the inventive hybrid plant of increased fertility. The method of the invention comprises 5 introducing a fertility restorer gene by genetic engineering and placing two or more copies of a fertility restorer gene at two or more gene loci which do not have a complete linkage relationship.

[0028] Without imposing any limitation, the invention 10 is preferably a method for producing comprises

1) introducing a fertility restorer gene by genetic engineering to produce a plant of fertility restoring line containing the fertility restorer genes homozygously at two or more loci; and

15 2) crossing the plant of fertility restoring line produced by the step of 1) with a plant of sterility line.

[0029] In step 1), the method of introducing the fertility recovery gene to the plant is not subject to any particular limitation; a known method that is suitable for 20 the type of plant may be used. Any suitable expression system for transduction by a genetic engineering technique can be employed. Recombinant expression vectors are composed of a nucleic acid containing a fertility restorer gene that can be introduced into the plant (e.g., rice Rf-1) and is operably linked to suitable transcriptional or translational regulatory base sequences, such as ones derived from a mammalian, microbial, viral or insect gene.

[0030] Illustrative examples of regulatory sequences

include transcriptional promoters, operators and enhancers, mRNA ribosome binding sites, and appropriate sequences which control transcription and translation initiation and termination. The base sequences are linked so as to be
5 capable of functioning when the regulatory sequences are functionally associated with the DNA sequence. Thus, a promoter base sequence is operably linked to a DNA sequence if the promoter base sequence controls the transcription of the DNA sequence. The expression vectors
10 generally incorporate an origin of replication that confers the ability to replicate in a plant, and a selection gene for identifying the transformant. Any commonly used selectable marker may be employed by a standard method. Illustrative examples include genes
15 resistant to antibiotics such as tetracycline, ampicillin, kanamycin, neomycin, hygromycin or spectinomycin.
[0031] If necessary, a sequence encoding an appropriate signal peptide (native or heterologous) can be incorporated into the expression vectors. A DNA sequence
20 for a signal peptide (secretory leader) may be fused in frame to a nucleic acid sequence so that first the DNA is transcribed, then the mRNA is translated into a fusion protein containing the signal peptide.

Methods for integrating a DNA fragment of a gene
25 into a vector such as a plasmid are described in, e.g., Sambrook, J., and Russell, D.W.: Molecular Cloning, A Laboratory Manual (3rd edition), (New York: Cold Spring Harbor Laboratory Press, 2001). Commercially available

ligation kits (such as those available from Takara Co., Ltd.) can be conveniently used. The recombinant vectors (e.g. recombinant plasmids) thus obtained are transferred into the host plant cells.

- 5 [0032] Vectors can be conveniently prepared by linking a desired gene to a recombinant vector available in the art (e.g. plasmid DNA) by an ordinary method. Plant transforming vectors are especially useful for conferring a plant with fertility using a nucleic acid fragment of
- 10 the present invention. Vectors for plants are not specifically limited so far as they can express the gene of interest in plant cells to produce the desired protein. Examples include pBI221, pBI121 (Clontech), and vectors derived therefrom. Examples of vectors for transforming
- 15 monocotyledons in particular include pIG121Hm and pTOK233 (both from Hiei et al., Plant J. 6, 271-282 (1994)), and pSB424 (Komari et al., Plant J., 10, 165-174 (1996)).
- [0033] Transgenic plants can be prepared by replacing the β -glucuronidase (GUS) gene in the above vectors with a
- 20 nucleic acid fragment of the present invention so to construct a plant transforming vector, and introducing the vector into a plant. The plant transforming vector preferably comprises at least a promoter, a translation start codon, a desired gene (the nucleic acid sequence of
- 25 the fertility restorer gene, or a portion thereof), a translation stop codon and a terminator. It may also contain DNA encoding a signal peptide, an enhancer sequence, non-translated regions at the 5' and 3' ends of

the desired gene, and a selectable marker region, as appropriate. Promoters and terminators are not specifically limited so long as they are functional in plant cells. Examples of constitutive expression 5 promoters include the 35S promoter initially contained in the above vectors, as well as promoters for actin and ubiquitin genes.

[0034] Examples of suitable methods for introducing a plasmid into a host cell include those mentioned by 10 Sambrook, J. et al. (2001), such as the calcium phosphate method, calcium chloride/rubidium chloride method, electroporation, electroinjection, chemical treatment such as with PEG, and methods involving the use of a gene gun or the like. Plant cells can be transformed by, for 15 example, the leaf disc method (Science 227, 129 (1985)) or electroporation (Nature 319, 791 (1986)).

[0035] Examples of methods for transferring a gene into a plant include methods involving the use of Agrobacterium (Horsch et al., Science 227, 129 (1985); 20 Hiei et al., Plant J. 6, 271-282 (1994)), electroporation (Fromm et al., Nature 319, 791 (1986)), a PEG method (Paszkowski et al., EMBO J. 3, 2717 (1984)), microinjection (Crossway et al., Mol. Gen. Genet. 202, 179 (1986)), and particle bombardment (McCabe et al., 25 Bio/Technology 6, 923 (1988)). No particular limitation is imposed on the method used, insofar as it is suitable for introducing nucleic acid into the desired plant.

[0036] Illustrative, non-limiting examples of

Agrobacterium-mediated methods for establishing plant
(e.g., rice) restorer lines include those described in
Hiei et al., Plant J. 6, 271-282 (1994); Komari et al.,
Plant J. 10, 165-174 (1996); and Ditta et al., Proc. Natl.
5 Acad. Sci. USA 77, 7347-7351(1980).

[0037] First, a plasmid vector containing the nucleic acid fragment to be introduced is prepared. Suitable plasmid vectors include pSB11 and pSB22, plasmid maps for which are described in the above-referenced Komari et al.,
10 Plant J. 10, 165-174 (1996). Alternatively, those skilled in the art may themselves construct a suitable vector based on the foregoing plasmid vectors such as pSB11 or pSB22. In the reference examples described later in the specification, an intermediate vector pSB200 having a
15 hygromycin-resistant gene cassette was prepared based on pSB11 and used. Specifically, a nopaline synthase terminator (Tnos) was first fused to a ubiquitin promoter and a ubiquitin intron (PubI-ubiII). A hygromycin-resistant gene (HYG(R)) was inserted between ubiI and Tnos
20 on the resulting PubI-ubiII-Tnos complex to give a PubI-ubiII-HYG(R)-Tnos assembly. This assembly was fused to a HindIII/EcoRI fragment of pSB11 (Komari et al., supra) to give pKY205. Linker sequences for adding restriction enzyme sites NotI, NspV, EcoRV, KpnI, SacI and EcoRI were
25 inserted at HindIII sites upstream of PubI on this pKY205 to give a vector pSB200 having a hygromycin-resistant gene cassette.

[0038] Next, Escherichia coli cells (e.g. DH5 α , JM109,

MV1184, all commercially available from suppliers such as Takara) are transformed with the recombinant vector containing the introduced nucleic acid.

[0039] The resulting transformed *E. coli* cells are used to carry out triparental mating with an *Agrobacterium* strain, preferably in combination with a helper *E. coli* strain, according to the method of Ditta et al. (1980), for example. Suitable *Agrobacterium* strains include the *A. tumefaciens* strains LBA4404/pSB1, LBA4404/pNB1 and LBA4404/pSB3. Plasmid maps for all of these are described in the above-referenced Komari et al., Plant J. 10, 165-174 (1996) and may be used by those skilled in the art to construct a vector. Suitable helper *E. coli* strains include, but are not limited to, HB101/pRK2013 (available from Clontech). Although less common, it has been reported that pRK2073-carrying *E. coli* cells can be used as helper *E. coli* (Lemas et al., Plasmid 27, 161-163 (1992)).

[0040] Next, the *Agrobacterium* cells mated as intended are used to carry out the transformation of a male sterile plant such as rice according to, inter alia, the method of Hiei et al. (1994). The immature rice seeds required for transformation can be prepared by, for example, pollinating male-sterile rice with a japonica cultivar.

[0041] The restoration of fertility in transformed plants can be examined by, for example, seed fertility evaluation in standing plants about one month after heading. "Evaluation in standing plants" refers herein to

the examination of plants as grown in, typically, a field. Alternatively, a laboratory study may be conducted on the grain ripening percentage in the panicles of grain.

[0042] Although not subject to any particular
5 limitation, the preparation of a fertility restorer line plant that is homozygous for the fertility restorer gene at two or more loci by using a genetic engineering technique to introduce the fertility restorer gene may be carried out as follows.

10 [0043] First, DNA is extracted by a standard method from a transformant in which fertility has been restored as described above, and genomic Southern analysis is carried out. The probe used at this time is prepared from a portion of the introduced gene fragment. A plurality of
15 individuals having a single copy insertion are selected based on the results of analysis. Next, individuals homozygous for the introduced gene are selected from T_1 plants obtained by self-fertilization in each case (these are referred to below as "A plants" and "B plants").

20 Selection can be carried out by the above-mentioned genomic Southern analysis, or by means of a PCR marker designed from base sequence information in the vicinity of the locus of gene introduction. Individuals homozygously containing the fertility restorer gene at two gene loci
25 are selected from F_2 plants obtained by crossing the native restorer line with an A plant. The genotype of the fertility restorer gene derived from the native restorer line can be inferred by, for example, the method described

in International Disclosure No. WO 03/027290 A1. As noted above, the genotype of the fertility restorer gene derived from A plants can be inferred by genomic Southern analysis, or by using PCR markers.

5 [0044] A similar method was used to select, from among F₁ plants obtained by crossing of (a native restorer line / A individuals) / (a native restorer line / B individuals), individuals homozygously containing the fertility restorer gene originating from the native
10 restorer line, and heterozygously containing the fertility restorer genes originating from A plants and B plants. By selecting, from among F₂ plants obtained by the self-fertilization of selected individuals, those plants which homozygously contain fertility restorer genes originating
15 from A individuals and B individuals, plants which homozygously have the fertility restorer gene at three gene loci can be prepared.

[0045] Before and after each step, it is possible to verify the chromosomal locations at which extrinsic genes
20 have been inserted. An illustrative, non-limiting example of a method for verifying these chromosomal locations is described below.

[0046] A sequence not native to the host plant is inserted along with the fertility restorer gene. For
25 example, in the subsequently described examples, a nopaline synthetase terminator (Tnos) (Nos in Fig. 9) is incorporated together with the rice Rf-1 gene. The Tnos sequence is included in the cloning vector pBI121

(Accession No. AF485783) deposited with a public database (Genbank). In Example 3, Nos was used to identify the sites of gene insertion on the chromosomes. Specifically, a primer (e.g., NosF2 in Fig. 9) was prepared based on a known Nos base sequence, and a polymerase chain reaction (PCR) was carried out. The end base sequence of the resulting PCR-amplified product was analyzed and a homology search was carried out on the Genbank database, from which the sequence was found to match the complementary strand sequence on a genomic clone of a specific chromosome on rice (e.g., AP004007 in Fig. 9). To further confirm the presence of the inserted genes on specific chromosomes, two primers may be designed (e.g., No6F and No6R in Fig. 9) and the PCR carried out on the specific chromosomes. When a hybrid plant genome containing the inserted genes is used as the template, an amplification product cannot be obtained by the PCR, whereas fragments of the desired length are amplified when a plant genome without the inserted genes is used.

Conversely, if a single primer based on the base sequence for Nos (e.g., NosF2 in Fig. 9) and one primer of a primer pair based on the chromosomal sequence (e.g., Nos6R in Fig. 9), both of which are used to identify the chromosomal sites of the inserted genes, are employed as a primer pair, a fragment of the desired length is amplified when a hybrid plant genome containing the inserted genes is used as the template, whereas amplification product is not observed when a plant genome without the inserted

genes is used.

[0047] The above verification technique can be employed in plants for which all or part of the base sequence of the genome has been confirmed. For example, 5 the base sequences of the genomes for rice and maize are disclosed in databanks such as Genbank, EMBL and DDBJ.

[0048] In step 2) of the inventive method, the plant of fertility restorer line produced in step 1) is crossed with a plant of sterile line, thereby enabling the 10 inventive plant to be obtained.

[0049] After the cross has been performed, plants having two or more copies of the fertility restorer gene at two or more gene loci which do not have a complete linkage relationship can be selected. The presence of two 15 or more copies of the fertility restorer gene on the hybrid plant can be confirmed from the number and/or intensity of bands in a Southern analysis.

[0050] The present invention also comprises a plant of fertility restoring line homozygously containing 20 the fertility restorer genes at two or more loci produced in step 1). By crossing such a fertility restorer line plant with a desired male-sterile line, the hybrid plant of the invention can be obtained.

[0051] Fertility Restorer Gene Rf-1 for Rice BT-Type
25 Male-Sterile Cytoplasm

The present inventors have isolated and identified the fertility restorer gene Rf-1 for rice BT-type male-sterile cytoplasm (reference examples). In the

subsequently described examples of the invention, hybrid rice is prepared using the Rf-1 gene as a gametic fertility restorer gene. The present inventors are separately applying for patents on the Rf-1 gene. The 5 details are as follows.

[0052] Related Patent Applications Already Filed by the Inventors:

Japanese Patent Application No. 2002-345485;
International Disclosure No. WO 02/14506 A1
10 Japanese Patent Applications No. 2001-285247,
2001-309135 and 2002-185709; International Disclosure No.
WO 03/027290 A1

Japanese Patent Application No. 2002-197560;
International Patent Application No. PCT/JP03/03154
15 Firstly, the present inventors have restricted the Rf-1 locus to a very small region on chromosome 10. Based of the results, we developed PCR markers situated close to the Rf-1 locus and found a method for detecting the Rf-1 gene by utilizing the linkage of these PCR markers to the 20 Rf-1 locus. Specifically, making use of the fact that the locus for the Rf-1 gene lies between the loci for the PCR markers S12564 Tsp509I and C1361 MwoI on chromosome 10 in rice, a study is performed to determine whether the Rf-1 gene is present and individuals homozygous for the Rf-1 25 gene are selected by genotyping the novel PCR marker loci situated nearby. The present inventors previously filed a patent application for a method of detecting the Rf-1 gene as Japanese Patent Application No. 2000-247204, later

published as Japanese Patent Public Disclosure No. 2002-345485. Based on this Japanese patent application, an international patent application (PCT/JP01/07052) was also filed, and published as International Disclosure No.

5 WO 02/14506 A1. The entire contents of these patent applications are incorporated herein by reference.

[0053] In addition, as improvements to the method disclosed in Japanese Patent Application No. 2000-247204, the present inventors have filed Japanese Patent

10 Applications No. 2001-285247 (September 19, 2001), 2001-309135 (October 4, 2001) and 2002-185709 (June 26, 2002) in which the region of the Rf-1 locus containing the Rf-1 gene is further restricted. Based on the three foregoing Japanese patent applications, the present inventors have

15 also filed an international patent application (PCT/JP02/09429). Moreover, the present inventors have conducted further research and identified the Rf-1 gene, for which Japanese Patent Application No. 2002-197560 on July 5, 2002 was filed. Based on the latter application,

20 an international patent application (PCT/JP03/03154) was filed, and published as International Disclosure No.

WO 03/027290 A1. The entire contents of these patent applications are incorporated herein by reference.

[0054] In Japanese Patent Public Disclosure No. 2002-25 345485, the present inventors disclosed that the locus of the Rf-1 gene lies between the loci for the DNA markers S12564 Tsp509I and C1361 MwoI, and describe a RFLP-PCR marker that uses the same. In addition, based on the

close linkage between the Rf-1 locus and the DNA marker locus S12564 Tsp509I, the present inventors used chromosome walking and genetic analysis to search in the region between DNA marker loci S12564 Tsp509I and C1361 5 MwoI for regions linked to the Rf-1 gene. As a result, the present inventors restricted the Rf-1 gene-containing Rf-1 locus region to about 76 kb and successfully determined its entire base sequence.

- [0055] Specifically, in Japanese Patent Public Disclosure No. 2002-345485, linkage analyses on a population of 1042 individuals prepared by pollinating MS Koshihikari with MS-FR Koshihikari (heterozygous at the Rf-1 locus) revealed one recombinant between the Rf-1 and S12564 Tsp509I loci and two recombinants between the Rf-1 10 and C1361 MwoI loci. The present inventors added another 4103 individuals to the population, and carried out an analysis on a total of 5145 individuals. As a result, one recombinant between the Rf-1 and S12564 Tsp509I loci and six recombinants between the Rf-1 and C1361 MwoI loci were 15 newly discovered, bringing the total numbers of the respective recombinants to two and eight. These ten individuals were submitted to high-precision segregation analysis as recombinants very near the Rf-1 locus 20 (Reference example 1).
- [0056] The higher frequency with which recombinants appeared between the Rf-1 and C1361 MwoI loci (8 recombinants) as opposed to between the Rf-1 and S12564 25 Tsp509I loci (2 recombinants) means that, of the S12564

Tsp509I locus and the C1361 MwoI locus, the former is genetically closer to the Rf-1 locus. The genetic distance (expressed as the recombination rate in cM units) and physical distance (expressed as the number of base pairs, or bp) are not always proportional to each other, but if the genetic distance is short, it can generally be expected that the physical distance will also be short.

[0057] Accordingly, the present inventors decided to isolate the Rf-1 locus by carrying out chromosome walking starting at the S12564 Tsp509I locus (Reference example 2). Chromosome walking was performed on a genomic library constructed with the λ DASH II vector using genomic DNA from the indica cultivar IR24 and the japonica cultivar Asominori. IR24 is a cultivar which carries Rf-1, and Asominori is a cultivar which does not carry Rf-1. As a result of chromosome walking, the present inventors were able to prepare contigs (ordered sets of overlapping clones on a chromosome) covering a chromosomal region of about 76 kb from genomic clones of IR24, and went on to determine the entire base sequence (76363 bp) of this region.

[0058] Next, using in part the base sequence information thus acquired, the present inventors developed 12 new markers and performed a high-precision segregation analysis on the above-described ten recombinants very near the Rf-1 locus (Reference example 3). The results showed that a 65 kb sequence included in the above-described approximately 76 kb chromosomal region contains a sequence

which determines whether the Rf-1 gene has functionality. This region is covered by a contig consisting of 8 genomic clones. Each clone has a length of about 12 to 22 kb and has overlapping domains of at least 4.7 kb. Rice genes
5 are known to vary in length from short genes to long genes, but most are thought to have a length of no more than several kilobases. Hence, it is expected that at least one of these eight genomic clones should contain the full-length Rf-1 gene.

10 [0059] The present inventors further restricted the Rf-1 gene region in the above chromosomal region of about 76 kb and performed complementation tests to directly demonstrate the presence of a fertility restoring ability.

[0060] Specifically, ten partial fragments (each 10 to 15 21 kb) in the above 76 kb region were separately introduced into immature seeds of the male-sterile line MS Koshihikari by genetic engineering techniques (Fig. 5). Of the ten partial fragments used, eight were derived from the eight genomic clones previously obtained by chromosome 20 walking (XSE1, XSE7, XSF4, XSF20, XSG22, XSG16, XSG8 and XSH18 shown in Fig. 1 and described in Reference example 3). Complementation tests were also performed on fragments derived from the two clones XSF18 and XSX1. XSF18 is identical to XSF20 at the 5' and 3' ends

25 (respectively bases 20328 and 41921 of SEQ ID NO:1), but lacks the intermediate bases 33947 to 38591. This is because the clone XSF18 was initially isolated but found to have incurred the above deletion during amplification

after isolation; hence, the amplification step was carried out once again and the complete clone was isolated and named XSF20. XSX1 is a clone that was newly prepared from clones XSG8 and XSH18 by restriction enzyme treatment and 5 ligation so as to contain a sufficiently overlapping domain because the overlapping domain of these two original clones was relatively small (about 7 kb).

[0061] Because Rf-1 is a dominant gene, if the inserted fragment contains the entire Rf-1 gene, fertility 10 will be restored in primary transformants. In complementation tests, plants transformed with each fragment were evaluated for seed fertility. Seed fertility was found to be restored in those plants transformed with a 15.6 kb fragment (including bases 38538 15 to 54123 of SEQ ID NO:1) derived from the λ phage clone XSG16 (Reference example 4). Plants transformed with the other fragments were all sterile. These results showed that the above 15.6 kb fragment completely contains the Rf-1 gene. Moreover, a method for introducing the Rf-1 20 gene by genetic engineering techniques was provided and demonstrated to be effective.

[0062] To further specify the portion of the λ phage clone XSG16 that contains the Rf-1 gene, the present inventors conducted seed fertility studies by 25 complementary tests on fragments shorter than the above 15.6 kb fragment (which includes bases 38538 to 54123 of SEQ ID NO:1). As a result, the present inventors found that seed fertility was restored in plants transformed

with an 11.4 kb fragment derived from XSG16 (containing bases 42357 to 53743 of SEQ ID NO:1) (Reference example 4(2)). Seed fertility was also restored in plants transformed with an even shorter 6.8 kb fragment

5 (containing bases 42132 to 48883 of SEQ ID NO:1)

(Reference example 4(3)). These results showed that the above 6.8 kb fragment contains the Rf-1 gene.

[0063] Continuing our research further, the present inventors have identified the nucleic acid that has a 10 fertility restoring ability and also determined the amino acid sequence encoded thereby. Specifically, as described subsequently in Reference examples 5 and 6, first, DNA fragments corresponding to bases 43733 to 44038 and bases 48306 to 50226 of SEQ ID NO:1 were prepared using the PCR. 15 Using these two fragments as probes (probes P and Q), the present inventors screened a cDNA library constructed from a line obtained by introducing Rf-1 into Koshihikari. As a result, the end base sequences of six of the clones matched the sequence of XSG16. These clones were isolated 20 as Rf-1 gene-containing clones, and their base sequences were analyzed (SEQ ID NOS: 43 to 48).

[0064] All of these sequences (SEQ ID NOS. 43 to 48) encode a protein having amino acid sequence 1 to 791 (SEQ ID NO:49). Specifically, bases 215 to 2587 of SEQ ID 25 NO:43, bases 213 to 2585 of SEQ ID NO:44, bases 218 to 2590 of SEQ ID NO:45, bases 208 to 2580 of SEQ ID NO:46, bases 149 to 2521 of SEQ ID NO:47 and bases 225 to 2597 of SEQ ID NO:48 all encode the amino acid sequence 1 to 791

of SEQ ID NO:49. Moreover, the above base sequence corresponds to bases 43907 to 46279 of SEQ ID NO:1.

[0065] The amino acid sequence of SEQ. ID NO:49 was compared with the putative amino acid sequence for the 5 maize fertility restorer gene (Rf2) (Cui et al., 1996), whereupon the seven amino acid residues at the N terminus (Met-Ala-Arg-Arg-Ala-Ala-Ser) were found to agree. These seven amino acid residues are thought to be part of a mitochondrial targeting signal (Liu et al., 2001). Based 10 on these results, it appears that the cDNA isolated in this study is completely contained within the coding region of the Rf-1 gene. Aside from the foregoing region, no homology at the amino acid level was observed between the rice Rf-1 gene and the maize Rf-2 gene.

15 [0066] In addition, the cDNA sequences isolated in the present study were compared with the genome sequence of IR24 (SEQ ID NO:1), revealing the structures of the exons and introns for the Rf-1 gene (Fig. 7). This demonstrated that various transcription products of different splicing 20 modes and poly (A) addition sites are present together within an individual plant. No introns are present within the encoding region of the Rf-1 gene.

[0067] The present inventors also performed a complementation assay on the 6.8 kb fragment that restored 25 seed fertility in the complementation test in Reference example 4(3). Specifically, in Reference example 7, seed fertility was restored when a complementation test was conducted using a 4.2 kb fragment (bases 42132 to 46318 on

SEQ ID NO:1) containing the promoter region and the anticipated translation region of the RF-1 gene in the above-mentioned 6.8 kb fragment.

[0068] In addition, six new clones containing nucleic acid having a fertility restoring ability were obtained in Reference example 8. First, a PCR was performed using two different primers corresponding to bases 45522 to 45545 and bases 45955 to 45932 of SEQ ID NO:1 and using the genomic clone XSG16 of IR24 as the template, thereby giving a DNA fragment. Using this DNA fragment as probe R, plaque hybridization was then carried out together with above probe P. Six new clones (#7 to #12) were then obtained from the plaques that were positive for either of probe P and probe R. The results are shown in SEQ ID NOS: 54 to 59.

[0069] All of these sequences (SEQ ID NOS: 54 to 59) presumably encode the protein of amino acid sequence 1 to 791 (SE ID NO:49). Specifically, bases 229 to 2601 of SEQ ID NO:54, bases 175 to 2547 of SEQ ID NO:55, bases 227 to 2599 of SEQ ID NO:56, bases 220 to 2592 of SEQ ID NO:57, bases 174 to 2546 of SEQ ID NO:58 and bases 90 to 2462 of SEQ ID NO:59 all encode the amino acid sequence 1 to 791 of SEQ ID NO:49. Moreover the above base sequence corresponds to bases 43907 to 46279 of SEQ ID NO:1.

[0070] The cDNA sequences isolated this time were compared with the genome sequence of IR24 (SEQ ID NO:1 in Japanese Patent Application No. 2001-285247), revealing the exon and intron structures (Fig. 8). Three of these

isolated cDNA sequences contained no exons unrelated to the anticipated translation region and had only a single exon (#10 to #12; SEQ ID NOS: 57 to 59).

[0071] The nucleic acid containing a fertility restorer gene (Rf-1) locus is nucleic acid having the base sequence of SEQ ID NO:1 or a base sequence that is at least 70% identical to the base sequence of SEQ ID NO:1, and includes nucleic acid having a fertility restoring ability. Moreover, as mentioned in Reference example 4, 10 of the base sequences in SEQ ID NO:1, it was confirmed in particular that the Rf-1 gene is completely included in bases 38538 to 54123. The Rf-1 gene-containing region was further specified as preferably bases 38538 to 54123, more preferably bases 42357 to 53743, even more preferably 15 bases 42132 to 48883, and still more preferably bases 42132 to 46318, of SEQ ID NO:1.

[0072] The following regions were identified as nucleic acid containing the Rf-1 gene:

- [0073]
- a) bases 215 to 2587 of SEQ ID NO:43,
 - 20 b) bases 213 to 2585 of SEQ ID NO:44,
 - c) bases 218 to 2590 of SEQ ID NO:45,
 - d) bases 208 to 2580 of SEQ ID NO:46,
 - e) bases 149 to 2521 of SEQ ID NO:47,
 - f) bases 225 to 2597 of SEQ ID NO:48,
 - 25 g) bases 229 to 2601 of SEQ ID NO:54,
 - i) bases 175 to 2547 of SEQ ID NO:55,
 - j) bases 227 to 2599 of SEQ ID NO:56,
 - k) bases 220 to 2592 of SEQ ID NO:57,

- 1) bases 174 to 2546 of SEQ ID NO:58, and
- m) bases 90 to 2462 of SEQ ID NO:59,

[0074] The above base sequences correspond to g) bases 43907 to 46279 of SEQ ID NO:1, and all encode the amino acid sequence 1 to 791 of SEQ ID NO:49.

[0075] In this specification, depending on the context, the phrase "base sequence of SEQ ID NO:1" refers to all of SEQ ID NO:1, or to a portion thereof which takes part in fertility restoring ability, particularly bases 38538 to 10 54123. It refers more preferably to bases 42357 to 53743, even more preferably to bases 42132 to 48883, and still more preferably to bases 42132 to 46318. It refers most preferably to g) bases 43907 to 46279 of SEQ ID NO:1, or to any one of a) bases 215 to 2587 of SEQ ID NO:43, b) 15 bases 213 to 2585 of SEQ ID NO:44, c) bases 218 to 2590 of SEQ ID NO:45, d) bases 208 to 2580 of SEQ ID NO:46, e) bases 149 to 2521 of SEQ ID NO:47, f) bases 225 to 2597 of SEQ ID NO:48, h) bases 229 to 2601 of SEQ ID NO:54, i) bases 175 to 2547 of SEQ ID NO:55, j) bases 227 to 2599 of 20 SEQ ID NO:56, k) bases 220 to 2592 of SEQ ID NO:57, l) bases 174 to 2546 of SEQ ID NO:58, and m) bases 90 to 2462 of SEQ ID NO:59 which corresponds thereto.

[0076] In the reference examples below, as the nucleic acid containing a fertility restorer gene (Rf-1), nucleic acid was isolated from a genomic library of indica rice 25 IR24 containing the Rf-1 gene and was determined to have the base sequence of SEQ ID NO:1. However, the nucleic acid containing a fertility restorer gene (Rf-1) of the

present invention can be derived, without particular limitation, from any indica variety carrying the Rf-1 gene. Illustrative examples of indica varieties carrying the Rf-1 gene include IR24, IR8, IR36, IR64, Chinsurah and BoroII.

5 Japonica varieties which do not carry the Rf-1 gene include, but are not limited to, Asominori, Koshihikari, Kirara 397, Akihikari, Akitakomachi, Sasanishiki, Kinuhikari, Nipponbare, Hatusuboshi, Koganebare, Hinohikari, Mineasahi, Aichinokaori, Hatsushima, Akebono, Fujihikari,

10 Minenoyukimochi, Kokonoemochi, Fukuhibiki, Dontokoi, Gohyakumangoku, Hanaechizen, Todorokiwase, Haenuki, Domannaka and Yamahikari. "Indica varieties" and "japonica varieties" are terms familiar to those skilled in the art, and so it will be readily apparent to those

15 conversant in the art which rice cultivars are encompassed by the present invention.

[0077] Nucleic acids that may be used in the present invention include genomic DNA (including corresponding cDNA), chemically synthesized DNA, DNA amplified by the

20 PCR, and combinations thereof.

Nucleic acids containing the Rf-1 gene of the present invention preferably have the base sequence of SEQ ID NO:1. At least one codon may encode the same amino acid; this is called degeneracy of the genetic code.

25 Hence, a DNA sequence not completely identical to SEQ ID NO:1 may encode a protein having an amino acid sequence completely identical to that encoded by SEQ ID NO:1. Such a variant DNA sequence may result from silent mutation

(e.g., occurring during PCR amplification), or can be a product of the deliberate mutagenesis of a native sequence.

[0078] The Rf-1 gene preferably encodes the amino acid sequence in SEQ ID NO:49, but is not limited to this sequence, and may instead code for a similar amino acid sequence having one or more amino acid deletion, addition or substitution.

All homologous proteins are included, provided they have a fertility restoring ability. There may be one or 10 more "amino acid variations," the number of such variations being preferably from 1 to 20, more preferably from 1 to 10, and most preferably from 1 to 5. The amino acid sequence which encodes the Rf-1 gene is at least about 70%, preferably at least about 80%, more preferably 15 at least about 90%, even more preferably at least about 95%, and most preferably at least about 98% identical with the amino acid sequence of SEQ ID NO:49.

[0079] The percent identity of the amino acid sequence may be determined by visual inspection and mathematical 20 calculation. Alternatively, the percent identity between two protein sequences may be determined based on the algorithm of S.B. Needleman and C.D. Wunsch (J. Mol. Biol. 48, 443-453 (1970), and by comparing sequence information using the GAP computer program available from the 25 University of Wisconsin Genetics Computer Group (UWGCG). The preferred default parameters for the GAP program include: (1) a scoring matrix such as blosum62 mentioned by S. Henikoff and J.G. Henikoff (Proc. Natl. Acad. Sci.

USA 89, 10915-10919 (1992)); (2) gap penalty of 12; (3) gap length penalty of 4; (4) no penalty for end gaps.

[0080] Use can also be made of other sequence comparison programs employed by those skilled in the art.

5 The percent identity can be determined by comparing sequence information using the BLAST program described by Altschul et al. (Nucl. Acids. Res. 25, 3389-3402 (1997). This program can be used from the National Center for Biotechnology Information (NCBI) or DNA Data Bank of Japan 10 (DDBJ) web site on the Internet. Various parameters for homology searches using the BLAST program are described in detail at the same web sites. Some of the settings can be changed as appropriate, although the searches are generally conducted using default values.

15 [0081] It is a well-known fact among those skilled in the art that, even among proteins having the same function, there may exist differences in the amino acid sequences depending on the cultivars from which they are derived. The Rf-1 gene, so long as it has a fertility restoring 20 ability, also includes such homologs and variants of the base sequence of SEQ ID NO:1. Here, "has a fertility restoring ability" means to confer fertility to rice plants or seeds when a DNA fragment has been inserted. The restoration of fertility may rely on protein 25 expression by the Rf-1 gene, or the nucleic acid (DNA or RNA) of the Rf-1 gene may itself play some function in the conferring of fertility.

[0082] In a non-limiting example of a method which may

be used to determine whether a homolog or variant of the Rf-1 gene functions to restore fertility, the nucleic acid fragment of interest is introduced into immature seeds obtained by pollinating MS Koshihikari (sterile line) with 5 Koshihikari according to the method of Hiei et al. (Plant Journal 6, No. 2, 272-282 (1994)). When the resulting transformants are grown under normal conditions, the seeds mature only if the inserted nucleic acid fragment has a fertility restoring ability.

10 [0083] The nucleic acid derived from a corresponding region of japonica Asominori not carrying the Rf-1 gene has the base sequence shown in SEQ ID NO:2. Corresponding parts of SEQ ID NO:2 and SEQ ID NO:1 have an overall identity of about 98%. Thus, nucleic acids containing the 15 locus of the fertility restorer gene (Rf-1) are at least about 70%, preferably at least about 80%, more preferably at least about 90%, still more preferably at least about 95%, and most preferably at least about 98% identical to SEQ ID NO:1. "SEQ ID NO:1" is most preferably g) bases 20 43907 to 46279 of SEQ ID NO:1 or any one of a) bases 215 to 2587 of SEQ ID NO:43, b) bases 213 to 2585 of SEQ ID NO:44, c) bases 218 to 2590 of SEQ ID NO:45, d) bases 208 to 2580 of SEQ ID NO:46, e) bases 149 to 2521 of SEQ ID NO:47, f) bases 225 to 2597 of SEQ ID NO:48, h) bases 229 25 to 2601 of SEQ ID NO:54, i) bases 175 to 2547 of SEQ ID NO:55, j) bases 227 to 2599 of SEQ ID NO:56, k) bases 220 to 2592 of SEQ ID NO:57, l) bases 174 to 2546 of SEQ ID NO:58, and m) bases 90 to 2462 of SEQ ID NO:59 which

corresponds thereto.

[0084] The percent identity of the nucleic acid may be determined by visual inspection and mathematical calculation. Alternatively, the percent identity between 5 two nucleic acid sequences can be determined by comparing sequence information using the GAP computer program, version 6.0, described by Devereux et al. in Nucl. Acids Res. 12, 387 (1984) and available from the University of Wisconsin Genetics Computer Group (UWGCG). The preferred 10 default parameters for the GAP program include: (1) a unary comparison matrix (containing a value of 1 for identities and 0 for non-identities) for nucleotides, and the weighted comparison matrix of Gribskov and Burgess, Nucl. Acids Res. 14, 6745 (1986), as described by Schwartz 15 and Dayhoff, eds., Atlas of Protein Sequence and Structure (National Biomedical Research Foundation, 1979), pp. 353-358; (2) a penalty of 3.0 for each gap and an additional 0.10 penalty for each symbol in each gap; and (3) no penalty for end gaps. Other sequence comparison programs 20 used by those skilled in the art may also be employed.

[0085] Preferred nucleic acids of the present invention also include nucleic acids which are capable of hybridizing to the base sequence of SEQ ID NO:1 under moderately stringent conditions and have a fertility 25 restoring ability, and nucleic acids which are capable of hybridizing to the base sequence of SEQ ID NO:1 under highly stringent conditions and have a fertility restoring ability.

[0086] As used herein, conditions of moderate stringency can be readily determined by those of ordinary skill in the art based on, for example, the length of the DNA. The basic conditions are set forth by Sambrook et al.

5 in Molecular Cloning: A Laboratory Manual, 2nd Ed., Vol. 1 (Cold Spring Harbor Laboratory Press, 1989), pp. 1.101-104, and include the use of a prewashing solution for nitrocellulose filters of 5x SSC, 0.5% SDS, 1.0 mM EDTA (pH 8.0); hybridization conditions of about 1x SSC to 6x

10 SSC at about 40°C to 60°C (or some other similar hybridization solution such as Stark's solution within approximately 50% formamide at about 42°C); and washing conditions of about 60°C, 0.5x SSC and 0.1% SDS. The hybridization temperature is about 15 to 20°C lower when

15 the hybridization solution contains about 50% formamide. Conditions of high stringency can also be readily determined by one skilled in the art based on, for example, the length of the DNA. Highly stringent conditions generally include hybridization and/or washing conditions

20 at higher temperature and/or lower salt concentration than the moderately stringent conditions described above. For example, such conditions include hybridization conditions of 0.1x SSC to 0.2x SSC at about 60 to 65°C and/or washing conditions of 0.2x SSC and 0.1% SDS at about 65 to 68°C.

25 It will be recognized by those skilled in the art that the temperature and the salt concentrations of the washing solution may be adjusted if necessary according to such factors as the length of the probe.

[0087] "SEQ ID NO:1" is most preferably g) bases 43907 to 46279 of SEQ ID NO:1 or any one of a) bases 215 to 2587 of SEQ ID NO:43, b) bases 213 to 2585 of SEQ ID NO:44, c) bases 218 to 2590 of SEQ ID NO:45, d) bases 208 to 2580 of 5 SEQ ID NO:46, e) bases 149 to 2521 of SEQ ID NO:47, f) bases 225 to 2597 of SEQ ID NO:48, h) bases 229 to 2601 of SEQ ID NO:54, i) bases 175 to 2547 of SEQ ID NO:55, j) bases 227 to 2599 of SEQ ID NO:56, k) bases 220 to 2592 of SEQ ID NO:57, l) bases 174 to 2546 of SEQ ID NO:58, and m) 10 bases 90 to 2462 of SEQ ID NO:59 which corresponds thereto.

[0088] The DNA of the invention also includes nucleic acids that differ from the base sequence of SEQ ID NO:1 on account of the deletion, insertion or substitution of one or more base, yet retain a fertility restoring ability.

15 Insofar as a fertility restoring ability is retained, the number of bases that are deleted, inserted or substituted is not subject to any particular limitation, although the number of such bases is preferably from 1 to several thousand, more preferably from 1 to 1,000, even more 20 preferably from 1 to 500, more preferably yet from 1 to 200, and most preferably from 1 to 100.

[0089] Once the Rf-1 gene is further specified based on the descriptions provided herein, one skilled in the art will be able to use the nucleic acid exclusive of 25 regions other than the Rf-1 gene and exclusive of intron regions within the Rf-1 gene. Given amino acids (particularly amino acid sequences in SEQ ID NO:49) may be substituted with, for example, residues having similar

physiochemical characteristics. Examples of such conservative substitutions include changes from one aliphatic residue to another, such as substitutions among Ile, Val, Leu and Ala; changes from one polar residue to another, such as substitutions between Lys and Arg, Glu and Asp, or Gln and Asn; and changes from one aromatic residue to another, such as substitutions among Phe, Trp and Tyr. Other well-known conservative substitutions include changes between entire regions having similar hydrophobic characteristics. Those skilled in the art will be capable of introducing desired deletions, insertions or substitutions using familiar gene engineering techniques, such as site-specific mutagenesis as described in Sambrook et al. (2001, *supra*).

15 [0090] The present inventors compared an indica variety IR24 (SEQ ID NO:27) that carries the Rf-1 gene with the japonica variety Asominori (SEQ ID NO:28) and a Nipponbare BAC clone deposited with GenBank (Accession No. AC068923), both of which do not carry it. As a result, 20 the present inventors found that the Rf-1 region of the indica variety which includes the Rf-1 gene has at least the following single-base polymorphisms (SNP):

[0091] 1) a base corresponding to base 1239 of SEQ ID NO:1 is A;

25 2) a base corresponding to base 6227 of SEQ ID NO:1 is A;

3) a base corresponding to base 20680 of SEQ ID NO:1 is G;

- 4) a base corresponding to base 45461 of SEQ ID
NO:1 is A;
 - 5) a base corresponding to base 49609 of SEQ ID
NO:1 is A;
 - 6) a base corresponding to base 56368 of SEQ ID
NO:1 is T;
 - 7) a base corresponding to base 57629 of SEQ ID
NO:1 is C; and
 - 8) a base corresponding to base 66267 of SEQ ID
- 10 NO:1 is G.
- [0092] Thus, nucleic acids containing the Rf-1 region of the present invention preferably meet anywhere from one to all of above conditions 1) to 8).
- [0093] In Reference example 3 below, the chromosomal organization of the Rf-1 region was examined for recombinants very near the Rf-1 gene (RS1 and RS2, RC1 to RC8). The results showed that a sequence determinative for the presence or absence of Rf-1 gene function is included in the sequence of bases 1239 to 66267 in SEQ ID
20 NO:1, i.e. in a region estimated to extend at most from the P4497 MboI locus to the B56691 XbaI locus (about 65 kb) (Fig. 3). However, there is a possibility that part of the genotype of the Rf-1 gene is important for the expression of genetic function by the Rf-1 gene in indica
25 varieties, while the remainder of the genotype gives rise to little difference in genetic function both in japonica varieties and indica varieties. In extreme cases, it may even be possible for the encoding region to be completely

identical in japonica and indica, with only the promoter regions differing, and for only part of the promoter regions and encoding regions to be included in the above region from the P4497 MboI locus to the B56691 XbaI locus
5 (approx. 65 kb). Therefore, it cannot be categorically stated that the above-described common indica region (bases 1239 to 66267 of SEQ ID NO:1) include the entire Rf-1 gene. Nonetheless, it does appear for the following reasons that at least SEQ ID NO:1 includes the Rf-1 gene
10 in its entirety:

- 1) a gene is generally several kilobases in size, and rarely exceeds 10 kb;
 - 2) the genomic base sequence of IR24 (SEQ ID NO:1) completely contains the common indica region above;
 - 15 3) the 5' end of SEQ ID NO:1 is located 1238 bp upstream of the 5' end of the above common indica region and forms a part of another gene (S12564); and
 - 4) the 3' end of SEQ ID NO:1 is located 10096 bp downstream of the 3' end of the above common indica region.
- 20 [0094] Moreover, the present inventors have confirmed from complementation tests that the Rf-1 gene is completely contained within, of the base sequence of SEQ ID NO:1, particularly bases 38538 to 54123. Therefore, in one embodiment of the invention, a base sequence which is
25 at least 70% identical to the base sequence of SEQ ID NO:1 or with the base sequence of bases 38538 to 54123 in SEQ ID NO:1 satisfies at least one of the following conditions
1) and 2):

1) a base corresponding to base 45461 of SEQ ID NO:1
is A; and

2) a base corresponding to base 49609 of SEQ ID NO:1
is A.

5 [0095] In addition, the present inventors have
identified the following regions as the nucleic acid
containing RF-1 gene:

- [0096] a) bases 215 to 2587 of SEQ ID NO:43,
b) bases 213 to 2585 of SEQ ID NO:44,
10 c) bases 218 to 2590 of SEQ ID NO:45,
d) bases 208 to 2580 of SEQ ID NO:46,
e) bases 149 to 2521 of SEQ ID NO:47,
f) bases 225 to 2597 of SEQ ID NO:48,
h) bases 229 to 2601 of SEQ ID NO:54,
15 i) bases 175 to 2547 of SEQ ID NO:55,
j) bases 227 to 2599 of SEQ ID NO:56,
k) bases 220 to 2592 of SEQ ID NO:57,
l) bases 174 to 2546 of SEQ ID NO:58, and
m) bases 90 to 2462 of SEQ ID NO:59,

20 [0097] The above base sequence corresponds to g) bases
43907 to 46279 of SEQ ID NO:1. Preferred nucleic acids of
the invention are:

- 25 n) nucleic acids which are at least 70% identical to
the nucleic acid of any one of regions a) to m) above, and
which have a fertility restoring ability;
o) nucleic acids which hybridize to the nucleic acid
of any one of regions a) to m) above under conditions that
are moderately or highly stringent, and which have a

fertility restoring ability; and

p) nucleic acids which are obtained by the deletion, insertion or substitution of one or more base in any one of regions a) to m) above, and which have a fertility 5 restoring ability.

[0098] Base 45461 of above SEQ ID NO:1 corresponds to
1) base 1769 of SEQ ID NO:43, 2) base 1767 of SEQ ID NO:44,
3) base 1772 of SEQ ID NO:45, 4) base 1762 of SEQ ID NO:46,
5) base 1703 of SEQ ID NO:47, 6) base 1779 of SEQ ID NO:48,
10 7) base 1783 of SEQ ID NO:54, 8) base 1729 of SEQ ID NO:55,
9) base 1781 of SEQ ID NO:56, 10) base 1774 of SEQ ID
NO:57, 11) base 1728 of SEQ ID NO:58 and 12) base 1644 of
SEQ ID NO:59. Therefore, it is especially preferable for
the nucleic acid used in the method of the invention to
15 satisfy at least one of the following conditions 1) to
12):

- 1) a base corresponding to base 1769 of SEQ ID NO:43
is A;
- 2) a base corresponding to base 1767 of SEQ ID NO:44
20 is A;
- 3) a base corresponding to base 1772 of SEQ ID NO:45
is A;
- 4) a base corresponding to base 1762 of SEQ ID NO:46
is A;
- 25 5) a base corresponding to base 1703 of SEQ ID NO:47
is A;
- 6) a base corresponding to base 1779 of SEQ ID NO:48
is A;

- 7) a base corresponding to base 1783 of SEQ ID NO:54
is A;
- 8) a base corresponding to base 1729 of SEQ ID NO:55
is A;
- 5 9) a base corresponding to base 1781 of SEQ ID NO:56
is A;
- 10 10) a base corresponding to base 1774 of SEQ ID
NO:57 is A;
- 11) a base corresponding to base 1728 of SEQ ID
10 NO:58 is A; or
- 12) a base corresponding to base 1644 of SEQ ID
NO:59 is A.
- [0099] In the complementation tests described in
Reference examples 4 and 7 of the specification, MS
15 Koshihikari (containing BT cytoplasm and having
substantially the same nuclear genes as Koshihikari) was
transformed by a method which uses the fragments derived
from the ten clones shown in Fig. 5 and uses Agrobacterium.
As a result, it was demonstrated that the fertility
20 restorer line is bred using nucleic acid containing the
base sequence of bases 38538 to 54123, preferably bases
42357 to 53743, more preferably bases 42132 to 48883, and
even more preferably bases 42132 to 46318, of SEQ ID NO:1.
- [0100] It was confirmed in the examples of the
25 invention that pollen fertility can be obtained using a
15.6 kb fragment derived from XSG16 as the Rf-1 gene. It
will be readily apparent to one conversant in the art that
longer fragments containing the foregoing fragment and, as

described above, shorter fragments which have been identified as containing the Rf-1 gene can likewise be used. The use of shorter fragments is preferred.

BRIEF DESCRIPTION OF THE DRAWINGS

5 [0101] [Fig. 1] Fig. 1 shows the results of chromosomal walking started from the RFLP marker locus S12564.

[Fig. 2] Fig. 2 shows an alignment of lambda clone contigs in relation to the BAC clone AC068923.

10 [Fig. 3] Fig. 3 shows the chromosomal organization of recombinant pollens proximal to the Rf-1 locus (all fertile) as mapped in close proximity to the Rf-1 locus based on the genotypes at the marker loci of 10 individuals (RS1, RS2, RC1-8) generated from the pollens.
15 White bars represent japonica regions and black bars represent indica regions.

[Fig. 4] Fig. 4 is a gene map in which the locus of Rf-1 gene on chromosome 10 of rice is positioned on a linkage map in relation to various markers; the values of
20 map distance were calculated from the segregation data from 1042 F1 individuals.

[Fig. 5] Fig. 5 shows fragments from 10 genomic clones used for the identification of the Rf-1 region by complementation assays. Lambda clones obtained by
25 chromosomal walking (thin lines) were used for complementation assays of the chromosomal regions shown by bold lines. XSF18 was found to contain a deletion shown by dotted line.

[Fig. 6] Fig. 6 shows the results of complementation assays using a 15.7 kb fragment from XSG16 (Reference example 4) and a 16.2 kb fragment from XSF18 (comprising bases 21065-33946 and 38592-41921 of SEQ ID NO:1). The plant transformed with the 15.7 kb fragment from XSG16 has restored fertility as proved by ears bowing.

[Fig. 7] Fig. 7 is a schematic of the Rf-1 gene structure. The white rods and black lines respectively indicate exon regions and intron regions. The number of base pairs for the exon regions are shown.

[Fig. 8] Fig. 8 is a schematic showing the relative positions of IR24 genome fragments on which a complementation assay was performed, the probes used in cDNA library screening, and the putative Rf-1 gene from the isolated cDNA. The white rods and black lines on the Rf-1 gene respectively indicate exon regions and intron regions. The number of base pairs for the exon regions are shown.

[Fig. 9] Fig. 9 is a schematic showing the position of primers used for verifying the site of Rf-1 insertion. Nos: nopaline synthase terminator (Tnos); HPT: hygromycin-resistant gene; BR: right border; BL: left border.

[Fig. 10] Fig. 10 is a schematic showing examples of methods for creating hybrid plants according to the invention and according to the prior art.

Examples

[0102] The following examples further illustrate the

present invention but are not intended to limit the technical scope thereof. Those skilled in the art will readily be capable of making various modifications and changes to the present invention based on the description provided herein. Such modifications and changes are encompassed within the technical scope of the invention.

[0103] Reference Examples

The following reference examples describe isolation and identification of the rice restorer gene for BT-type male sterility, as well as confirmation of the activity to restore fertility.

[0104] Reference example 1: Acquisition of recombinant individuals proximal to the Rf-1 locus
(Materials and Methods)

DNA was extracted from each of 4103 individuals of BC10F1 population produced by pollinating MS Koshihikari (generation: BC10F1) with MS-FR Koshihikari (generation: BC9F1, heterozygous at the Rf-1 locus), and genotyped at the S12564 Tsp509I and C1361 MwoI loci in the same manner as described in Reference example 2 above. Individuals having a genotype homozygous for Koshihikari at the S12564 Tsp509I locus were regarded as those generated by recombination between the Rf-1 and S12564 Tsp509I loci, while individuals having a genotype homozygous for Koshihikari at the C1361 MwoI locus were regarded as those generated by recombination between the Rf-1 and C1361 MwoI loci.

[0105] (Results and Discussion)

A survey of 4103 individuals revealed one recombinant individual between the Rf-1 and S12564 Tsp509I loci and 6 recombinant individuals between the Rf-1 and C1361 MwoI loci. The previous survey of 1042 individuals obtained by 5 crossing in Reference example 2 above had already revealed one recombinant individual between the Rf-1 and S12564 Tsp509I loci and 2 recombinant individuals between the Rf-1 and C1361 MwoI loci as shown in Table 3.

[0106] Thus, a total of 2 recombinant individuals 10 between the Rf-1 and S12564 Tsp509I loci and 8 recombinant individuals between the Rf-1 and C1361 MwoI loci were able to be obtained from 5145 individuals. These 10 individuals were tested by high-precision segregation analysis in the reference examples below.

15 [0107] Reference example 2: chromosomal walking

(1) First chromosomal walking

(Materials and Methods)

A genomic library was constructed from the genomic DNA of Asominori japonica (not carrying Rf-1) using Lambda 20 DASH II vector as described in Reference example 1 and tested by chromosomal walking.

[0108] PCR was routinely performed using total DNA of Asominori as a template in combination with the following primer pair:

25 5'-atcaggaggccttcaaattggaaac-3' (SEQ ID NO:3) and
5'-ctcgcaattgtttaatttgacc-3' (SEQ ID NO:4)
designed for a partial base sequence (Accession No.
D47284) of RFLP probe S12564. The resulting amplification

products of about 1200 bp were electrophoresed on an agarose gel and then purified by QIAEXII (QIAGEN). The purified DNA was labeled with a rediprime DNA labelling system (Amersham Pharmacia) to give a library screening probe (probe A, Fig. 1).

[0109] The library was routinely screened after plaques were blotted onto Hybond-N⁺ (Amersham Pharmacia). Single plaques were separated, after which phage DNA was purified by the plate lysate method using Lambda Midi kit [0110] (QIAGEN).

(Results and Discussion)

The results of terminal base sequence analysis and restriction enzyme fragment length analysis showed that two (WSA1 and WSA3) of 4 clones obtained by screening were 15 in a relative position as shown in Fig. 1. The Asominori genomic base sequences corresponding to WSA1 and WSA3 were determined by primer walking (DNA Sequencer 377, ABI).

[0111] (2) Second chromosomal walking

(Materials and Methods)

20 In addition to the Asominori genomic library described above, an IR24 genomic library was similarly constructed from the genomic DNA of an indica variety IR24 (carrying Rf-1) and tested by chromosomal walking.

[0112] PCR was routinely performed using DNA of WSA3 25 as a template in combination with the following primer pair:

5'-tgaaggagttatgggtgcgtgacg-3' (SEQ ID NO:5) and
5'-ttgccgagcacacttgccatgtgc-3' (SEQ ID NO:6)

designed for the Asominori genomic base sequence determined in (1). The resulting amplification products of 524 bp were purified and labeled by the method described above to give a library screening probe (probe E, 5 Fig. 1).

[0113] Library screening and phage DNA purification were performed by the method described above.

[0114] (Results and Discussion)

The results of terminal base sequence analysis and 10 restriction enzyme fragment length analysis showed that one (WSE8) of 15 clones obtained by screening of the Asominori genomic library was in a relative position as shown in Fig. 1. The Asominori genomic base sequence corresponding to WSE8 was determined by primer walking.

15 [0115] The results of terminal base sequence analysis and restriction enzyme fragment length analysis showed that two (XSE1 and XSE7) of 7 clones obtained by screening of the IR24 genomic library were in a relative position as shown in Fig. 1. The IR24 genomic base sequences 20 corresponding to XSE1 and XSE7 were determined by primer walking.

[0116] (3) Third chromosomal walking
(Materials and Methods)

The Asominori genomic library and IR24 genomic 25 library described above were tested by chromosomal walking.

[0117] PCR was routinely performed using DNA of WSE8 as a template in combination with the following primer pair:

5'-gcgacgcaatggacatagtgtcc-3' (SEQ ID NO:7) and
5'-ttacctgccaaggcaatatccatcg-3' (SEQ ID NO:8)
designed for the Asominori genomic base sequence
determined in (2). The resulting amplification products
5 of 1159 bp were purified and labeled by the method
described above to give a library screening probe (probe F,
Fig. 1).

[0118] Library screening and phage DNA purification
were performed by the method described above.

10 [0119] (Results and Discussion)

The results of terminal base sequence analysis and
restriction enzyme fragment length analysis showed that
two (WSF5 and WSF7) of 8 clones obtained by screening of
the Asominori genomic library were in a relative position
15 as shown in Fig. 1. The Asominori genomic base sequences
corresponding to WSF5 and WSF7 were determined by primer
walking.

[0120] The results of terminal base sequence analysis
and restriction enzyme fragment length analysis showed
20 that two (XSF4 and XSF20) of 13 clones obtained by
screening of the IR24 genomic library were in a relative
position as shown in Fig. 1. The IR24 genomic base
sequences corresponding to XSF4 and XSF20 were determined
by primer walking.

25 [0121] (4) Fourth chromosomal walking
(Materials and Methods)

The Asominori genomic library and IR24 genomic
library described above were tested by chromosomal walking.

[0122] PCR was routinely performed using DNA of WSF7 as a template in combination with the following primer pair:

5'-aaggcatactcagtggagggcaag-3' (SEQ ID NO:9) and
5'-ttaacctgaccgcAACGcacctgtc-3' (SEQ ID NO:10)
designed for the Asominori genomic base sequence determined in (3). The resulting amplification products of 456 bp were purified and labeled by the method described above to give a library screening probe (probe G,
10 Fig. 1).

[0123] Library screening and phage DNA purification were performed by the method described above.

[0124] (Results and Discussion)

The results of terminal base sequence analysis and
15 restriction enzyme fragment length analysis showed that two (WSG2 and WSG6) of 6 clones obtained by screening of the Asominori genomic library were in a relative position as shown in Fig. 1. The Asominori genomic base sequences corresponding to WSG2 and WSG6 were determined by primer
20 walking.

[0125] The results of terminal base sequence analysis and restriction enzyme fragment length analysis showed that three (XSG8, XSG16 and XSG22) of 14 clones obtained by screening of the IR24 genomic library were in a
25 relative position as shown in Fig. 1. The IR24 genomic base sequences corresponding to XSG8, XSG16 and XSG22 were determined by primer walking.

[0126] (5) Fifth chromosomal walking

(Materials and Methods)

The IR24 genomic library described above was tested by chromosomal walking.

[0127] The present inventors perused the public website of TIGR (The Institute for Genomic Research) and found that a BAC (Bacterial Artificial Chromosome) clone (Accession No. AC068923) containing RFLP marker S12564 had been deposited with a public database (GenBank). This BAC clone contains the genomic DNA of Nipponbare japonica and it was shown from base sequence comparison to completely include the contig regions of Asominori and IR24 prepared in (1)-(4) (Fig. 2).

[0128] Thus, PCR was routinely performed using total DNA of IR24 as a template in combination with the following primer pair:

5'-tggatggactatgtgggtcagtc-3' (SEQ ID NO:11) and
5'-agtggaaagtggagagagtagggag-3' (SEQ ID NO:12)
designed to amplify a part of this BAC clone. The resulting amplification products of about 600 bp were purified and labeled by the method described above to give a library screening probe (probe H, Fig. 1).

[0129] Library screening and phage DNA purification were performed by the method described above.

[0130] (Results and Discussion)

25 The results of terminal base sequence analysis and restriction enzyme fragment length analysis showed that one (XSH18) of 15 clones obtained by screening of the IR24 genomic library was in a relative position as shown in

Fig. 1. The IR24 genomic base sequence corresponding to XSH18 was determined by primer walking.

[0131] Reference example 3: High precision segregation analysis

5 (1) Development of PCR marker P4497 MboI Comparison between the genomic base sequence corresponding to the IR24 contig (SEQ ID NO:1) and the genomic base sequence corresponding to the Asominori contig (SEQ ID NO:2) determined in Reference example 2 revealed that the
10 1239th base of SEQ ID NO:1 is A while the 12631st base of SEQ ID NO:2 corresponding to said position is G.

[0132] For detecting this change, fragments of about 730 bp are first amplified by PCR from a region surrounding said position using the following primer pair:

15 P4497 MboI F:

5'-ccctccaacacataaatggtag-3' (SEQ ID NO:13)
(corresponding to bases 853-876 of SEQ ID NO:1)
(corresponding to bases 12247-12270 of SEQ ID NO:2)

and

20 P4497 MboI R:

5'-tttctgccaggaaactgttagatg-3' (SEQ ID NO:14)
(corresponding to bases 1583-1560 of SEQ ID NO:1)
(corresponding to bases 12975-12952 of SEQ ID NO:2).

The amplification products can be visualized by
25 electrophoresis on an agarose gel after treatment with MboI. Thus, the change can be detected as a difference in mobility in the agarose gel due to the difference in the length of DNA after MboI treatment because the

amplification products from Asominori DNA having an MboI recognition sequence (GATC) are cleaved with MboI while the amplification products from IR24 DNA are not cleaved with MboI for the lack of the MboI recognition sequence.

5 [0133] (2) Development of PCR marker P9493 BsII

Comparison between the genomic base sequence corresponding to the IR24 contig (SEQ ID NO:1) and the genomic base sequence corresponding to the Asominori contig (SEQ ID NO:2) determined in Reference example 2 10 revealed that the 6227th base of SEQ ID NO:1 is A while the 17627th base of SEQ ID NO:2 corresponding to said position is C.

[0134] For detecting this change, fragments of 126 bp are first amplified by PCR from a region surrounding said 15 position using the following primer pair:

P9493 BsII F:

5'-gcgatcttatacgcatactatgcg-3' (SEQ ID NO:15)

(corresponding to bases 6129-6152 of SEQ ID NO:1)

(corresponding to bases 17529-17552 of SEQ ID NO:2)

20 and

P9493 BsII R:

5'-aaagtcttgttccttcaccaagg-3' (SEQ ID NO:16)

(corresponding to bases 6254-6231 of SEQ ID NO:1)

(corresponding to bases 17654-17631 of SEQ ID NO:2).

25 The amplification products can be visualized by electrophoresis on an agarose gel after treatment with BsII. Thus, the change can be detected as a difference in mobility in the agarose gel due to the difference in the

length of DNA after BsII treatment because the amplification products from Asominori DNA having a BsII recognition sequence (CCNNNNNNNGG) are cleaved with BsII while the amplification products from IR24 DNA are not 5 cleaved with BsII for the lack of the BsII recognition sequence.

[0135] This marker was developed by applying the dCAPS method (Michaels and Amasino 1998, Neff et al., 1998). Specifically, g is substituted for a at the base 6236 of 10 SEQ ID NO:1 and the base 17636 of SEQ ID NO:2 by the use of P9493 BsII R primer described above. Thus, the fragments from Asominori DNA come to have a sequence of CCtttccttGG at 17626-17636 of SEQ ID NO:28 so that they are cleaved with BsII.

15 [0136] (3) Development of PCR marker P23945 MboI Comparison between the genomic base sequence corresponding to the IR24 contig (SEQ ID NO:1) and the genomic base sequence corresponding to the Asominori contig (SEQ ID NO:2) determined in Reference example 2 20 revealed that the 20680th base of SEQ ID NO:1 is G while the 32079th base of SEQ ID NO:2 corresponding to said position is A.

[0137] For detecting this change, fragments of 260 bp are first amplified by PCR from a region surrounding said 25 position using the following primer pair:

P23945 MboI F:

5'-gaggattatcaaaacaggatggacg-3' (SEQ ID NO:17)

(corresponding to bases 20519-20544 of SEQ ID NO:1)

(corresponding to bases 31918-31943 of SEQ ID NO:2)

and

P23945 MboI R:

5'-tgggcggcagcagtggaggataga-3' (SEQ ID NO:18)

5 (corresponding to bases 20778-20755 of SEQ ID NO:1)

 (corresponding to bases 32177-32154 of SEQ ID NO:2).

The amplification products can be visualized by electrophoresis on an agarose gel after treatment with MboI. Thus, the change can be detected as a difference in 10 mobility in the agarose gel due to the difference in the length of DNA after MboI treatment because the amplification products from IR24 DNA having an MboI recognition sequence (GATC) are cleaved with MboI while the amplification products from Asominori DNA are not 15 cleaved with MboI for the lack of the MboI recognition sequence.

[0138] (4) Development of PCR marker P41030 TaqI

Comparison between the genomic base sequence corresponding to the IR24 contig (SEQ ID NO:1) and the 20 genomic base sequence corresponding to the Asominori contig (SEQ ID NO:2) determined in Reference example 2 revealed that the 45461st base of SEQ ID NO:1 is A while the 49164th base of SEQ ID NO:2 corresponding to said position is G.

25 [0139] For detecting this change, fragments of 280 bp are first amplified by PCR from a region surrounding said position using the following primer pair:

P41030 TaqI F:

5'-aagaagggagggttatagaatctg-3' (SEQ ID NO:19)

(corresponding to bases 45369-45392 of SEQ ID NO:1)

(corresponding to bases 49072-49095 of SEQ ID NO:2)

and

5 P41030 TagI R:

5'-atacaggactaacaccactgctc-3' (SEQ ID NO:20)

(corresponding to bases 45648-45625 of SEQ ID NO:1)

(corresponding to bases 49351-49328 of SEQ ID NO:2).

The amplification products can be visualized by

10 electrophoresis on an agarose gel after treatment with TagI. Thus, the change can be detected as a difference in mobility in the agarose gel due to the difference in the length of DNA after TagI treatment because the amplification products from Asominori DNA having a TagI
15 recognition sequence (TCGA) are cleaved with TagI while the amplification products from IR24 DNA are not cleaved with TagI for the lack of the TagI recognition sequence.

[0140] (5) Development of PCR marker P45177 BstUI

Comparison between the genomic base sequence

20 corresponding to the IR24 contig (SEQ ID NO:1) and the genomic base sequence corresponding to the Asominori contig (SEQ ID NO:2) determined in Reference example 2 revealed that the 49609th base of SEQ ID NO:1 is A while the 53311st base of SEQ ID NO:2 corresponding to said
25 position is G.

[0141] For detecting this change, fragments of 812 bp are first amplified by PCR from a region surrounding said position using the following primer pair:

P45177 BstUI F:

5'-acgagtagtagcgatttccagcg-3' (SEQ ID NO:21)
(corresponding to bases 49355-49378 of SEQ ID NO:1)
(corresponding to bases 53057-53080 of SEQ ID NO:2)

5 and

P45177 BstUI R:

5'-cagcgtaaaaactaaaaacggaggc-3' (SEQ ID NO:22)
(corresponding to bases 50166-50143 of SEQ ID NO:1)
(corresponding to bases 53868-53845 of SEQ ID NO:2).

10 The amplification products can be visualized by
electrophoresis on an agarose gel after treatment with
BstUI. Thus, the change can be detected as a difference
in mobility in the agarose gel due to the difference in
the length of DNA after BstUI treatment because the
15 amplification products from IR24 DNA having a BstUI
recognition sequence (CGCG) at two positions are cleaved
into 3 fragments with BstUI while the amplification
products from Asominori DNA having the BstUI recognition
sequence at three positions are cleaved with BstUI into
20 four fragments.

[0142] (6) Development of PCR marker B60304 MspI
Comparison between the genomic base sequence
corresponding to the IR24 contig (SEQ ID NO:1) determined
in Reference example 2 and the base sequence of the BAC
25 clone described above (Accession No. AC068923) revealed
that the 56368th base of SEQ ID NO:1 is T while the base
of AC068923 corresponding to said position is C.

[0143] For detecting this change, fragments of about

330 bp are first amplified by PCR from a region surrounding said position using the following primer pair:

B60304 MspI F:

5'-atccccacatcatcataatccgacc-3' (SEQ ID NO:23)

5 (corresponding to bases 56149-56172 of SEQ ID NO:1)

and

B60304 MspI R:

5'-agtttcccttgatacggtgccg-3' (SEQ ID NO:24)

(corresponding to bases 56479-56455 of SEQ ID NO:1).

10 The amplification products can be visualized by electrophoresis on an agarose gel after treatment with MspI. Thus, the change can be detected as a difference in mobility in the agarose gel due to the difference in the length of DNA after MspI treatment because the

15 amplification products from Nipponbare DNA having an MspI recognition sequence (CCGG) are cleaved with MspI while the amplification products from IR24 DNA are not cleaved with MspI for the lack of the MspI recognition sequence.

[0144] This marker was developed by applying the dCAPS method. Specifically, t is substituted for g at base 56463 of SEQ ID NO:1 by the use of B60304 MspI R primer. As a result, the MspI recognition sequence of bases 56460-56463 of SEQ ID NO:1 changes from CCGG into ccgt so that the fragments from SEQ ID NO:1 become unable to be cleaved 25 with MspI. Thus, the fragments from IR24 have no MspI recognition sequence, while DNA from Nipponbare has the MspI recognition sequence at one position in a region corresponding to bases 56367-56370 of SEQ ID NO:1.

[0145] (7) Development of PCR marker B59066 BsaJI

Comparison between the genomic base sequence corresponding to the IR24 contig (SEQ ID NO:1) determined in Reference example 2 and the base sequence of the BAC 5 clone described above (Accession No. AC068923) revealed that the 57629th base of SEQ ID NO:1 is C while the base of AC068923 corresponding to said position is CC.

[0146] For detecting this change, fragments of about 420 bp are first amplified by PCR from a region 10 surrounding said position using the following primer pair:

B59066 BsaJI F:

5'-atttgttggtagttgcggctgag-3' (SEQ ID NO:25)

(corresponding to bases 57563-57586 of SEQ ID NO:1)

and

15 B59066 BsaJI R:

5'-gcccaaactcaaaaaggagagaacc-3' (SEQ ID NO:26)

(corresponding to bases 57983-57960 of SEQ ID NO:1).

The amplification products can be visualized by electrophoresis on an agarose gel after treatment with 20 BsaJI. Thus, the change can be detected as a difference in mobility in the agarose gel due to the difference in the length of DNA after BsaJI treatment because the amplification products from Nipponbare DNA having a BsaJI 25 recognition sequence (CCNNGG) are cleaved with BsaJI while the amplification products from IR24 DNA are not cleaved with BsaJI for the lack of the BsaJI recognition sequence.

[0147] (8) Development of PCR marker B56691 XbaI

Comparison between the genomic base sequence

corresponding to the IR24 contig (SEQ ID NO:1) determined in Reference example 2 and the base sequence of the BAC clone described above (Accession No. AC068923) revealed that the 66267th base of SEQ ID NO:1 is G while the base 5 of AC068923 corresponding to said position is C.

[0148] For detecting this change, fragments of about 670 bp are first amplified by PCR from a region surrounding said position using the following primer pair:
B56691 XbaI F:

10 5'-cctcaagtctccctaaaggcact-3' (SEQ ID NO:27)
(corresponding to bases 66129-66152 of SEQ ID NO:1)
and

B56691 XbaI R:

5'-gctctactgctgataaacccgttag-3' (SEQ ID NO:28)
15 (corresponding to bases 66799-66776 of SEQ ID NO:1).
The amplification products can be visualized by electrophoresis on an agarose gel after treatment with XbaI. Thus, the change can be detected as a difference in mobility in the agarose gel due to the difference in the 20 length of DNA after XbaI treatment because the amplification products from Nipponbare DNA having an XbaI recognition sequence (TCTAGA) are cleaved with XbaI while the amplification products from IR24 DNA are not cleaved with XbaI for the lack of the XbaI recognition sequence.

25 [0149] (9) Development of PCR marker B53627 BstZ17I
Comparison between the genomic base sequence corresponding to the IR24 contig (SEQ ID NO:1) determined in Reference example 2 and the base sequence of the BAC

clone described above (Accession No. AC068923) revealed that the 69331st base of SEQ ID NO:1 is T while the base of AC068923 corresponding to said position is C.

[0150] For detecting this change, fragments of about 5 620 bp are first amplified by PCR from a region surrounding said position using the following primer pair:
B53627 BstZ17I F:
5'-tggatggactatgtggggtcagtc-3' (SEQ ID NO:29)

(corresponding to bases 68965-68988 of SEQ ID NO:1)
10 and

B53627 BstZ17I R:
5'-agtggaagtggagagacttagggag-3' (SEQ ID NO:30)
(corresponding to bases 69582-69559 of SEQ ID NO:1).

The amplification products can be visualized by 15 electrophoresis on an agarose gel after treatment with BstZ17I. Thus, the change can be detected as a difference in mobility in the agarose gel due to the difference in the length of DNA after BstZ17I treatment because the amplification products from IR24 DNA having a BstZ17I 20 recognition sequence (GTATACT) are cleaved with BstZ17I while the amplification products from Nipponbare DNA are not cleaved with BstZ17I for the lack of the BstZ17I recognition sequence.

[0151] (10) Development of PCR marker B40936 MseI
25 Development of all the following PCR markers
(10)-(12) relates to a study of the base sequences corresponding to further downstream regions (3') of base 76363 at the 3'end of SEQ ID NO:1.

[0152] The following primer pair was designed for the base sequence of the BAC clone described above (Accession No. AC068923):

5'-tacgacgccatttcactccattgc-3' (SEQ ID NO:31)

5 and

5'-catttctctatggcggtgctctg-3' (SEQ ID NO:32).

PCR was routinely performed using this primer pair in combination with total DNAs of MS-FR Koshihikari (genotype of the Rf-1 locus: Rf-1 Rf-1) and Koshihikari as templates.

10 The resulting amplification products of about 1300 bp were electrophoresed on an agarose gel and then purified by QIAEXII (QIAGEN). Analysis of the base sequence of the purified DNA by a DNA sequencer 377 (ABI) showed several polymorphisms.

15 [0153] One of them can be detected by PCR amplification of a region surrounding said position using the following primer pair:

B40936 MseI F:

5'-acctgttaggttatggcaccttcaacac-3' (SEQ ID NO:33)

20 and

B40936 MseI R:

5'-ccaaggaaacgaagttcaaatgtatgg-3' (SEQ ID NO:34).

The amplification products can be visualized by electrophoresis on an agarose gel after treatment with

25 MseI. Thus, the change can be detected as a difference in mobility in the agarose gel due to the difference in the length of DNA after MseI treatment because the amplification products from MS-FR Koshihikari (Rf-1 Rf-1)

DNA having an MseI recognition sequence (TTAA) are cleaved with MseI while the amplification products from Koshihikari DNA are not cleaved with MseI for the lack of the MseI recognition sequence.

5 [0154] This marker was developed by applying the dCAPS method.

[0155] (11) Development of PCR marker B19839 MwoI

The following primer pair was designed for the base sequence of the BAC clone described above (Accession No.

10 AC068923):

5'-tgatgtttggcatcccttcg-3' (SEQ ID NO:35)

and

5'-gagatagggacgacagacacgc-3' (SEQ ID NO:36).

PCR was routinely performed using this primer pair in combination with total DNAs of MS-FR Koshihikari (genotype of the Rf-1 locus: Rf-1 Rf-1) and Koshihikari as templates. The resulting amplification products of about 1200 bp were electrophoresed on an agarose gel and then purified by QIAEXII (QIAGEN). Analysis of the base sequence of the 20 purified DNA by a DNA sequencer 377 (ABI) showed several polymorphisms.

[0156] One of them can be detected by PCR amplification of a region surrounding said position using the following primer pair:

25 B19839 MwoI F:

5'-tcctatggctgttttagaaaactgcaca-3' (SEQ ID NO:37)

and

B19839 MwoI R:

5'-caagttcaaacataactggcggtt-3' (SEQ ID NO:38).

The amplification products can be visualized by electrophoresis on an agarose gel after treatment with MwoI. Thus, the change can be detected as a difference in 5 mobility in the agarose gel due to the difference in the length of DNA after MwoI treatment because the amplification products from Koshihikari DNA having an MwoI recognition sequence (GCNNNNNNNGC) are cleaved with MwoI while the amplification products from MS-FR Koshihikari 10 (Rf-1 Rf-1) DNA are not cleaved with MwoI for the lack of the MwoI recognition sequence.

[0157] This marker was developed by applying the dCAPS method.

[0158] (12) Development of PCR marker B2387 BfaI
15 The following primer pair was designed for the base sequence of the BAC clone described above (Accession No. AC068923):

5'-cactgtccctgtaaagtgtgtgtgc-3' (SEQ ID NO:39)

and

20 5'-caagcgtgtataaaaatgtgacgc-3' (SEQ ID NO:40).

PCR was routinely performed using this primer pair in combination with total DNAs of MS-FR Koshihikari (genotype of the Rf-1 locus: Rf-1 Rf-1) and Koshihikari as templates. The resulting amplification products of about 1300 bp were 25 electrophoresed on an agarose gel and then purified by QIAEXII (QIAGEN). Analysis of the base sequence of the purified DNA by a DNA sequencer 377 (ABI) showed several polymorphisms.

[0159] One of them can be detected by PCR amplification of a region surrounding said position using the following primer pair:

B2387 BfaI F:

5 5'-tgcctactgccattactatgtgac-3' (SEQ ID NO:41)

and

B2387 BfaI R:

5'-acatactaccgtaaatggtctctg-3' (SEQ ID NO:42).

The amplification products can be visualized by
10 electrophoresis on an agarose gel after treatment with
BfaI. Thus, the change can be detected as a difference in
mobility in the agarose gel due to the difference in the
length of DNA after BfaI treatment because the
amplification products from Koshihikari DNA having an BfaI
15 recognition sequence (CTAG) are cleaved with BfaI while
the amplification products from MS-FR Koshihikari (Rf-1
Rf-1) DNA are not cleaved with BfaI for the lack of the
BfaI recognition sequence.

(13) Segregation analysis

20 Two recombinants between the Rf-1 and S12564 Tsp509I
loci (RS1 and RS2) and 8 recombinants between the Rf-1 and
C1361 MwoI loci (RC1 to RC8) obtained in Reference example
1 were genotyped at the 12 DNA marker loci developed in
(1) to (12) above. The results are shown in Table 1 along
25 with the genotypes of each recombinant at the S12564
Tsp509I and C1361 MwoI loci.

[0160] [Table 1]

Table 1 Genotypes of recombinants proximal to the Rf-1 locus at various marker loci

Locus	R51	R52	RC1	RC2	RC3	RC4	RC5	RC6	RC7	RC8
S12564 Tsp509I	J	J	H	H	H	H	H	H	H	H
P4497 MboI	J	J	H	H	H	H	H	H	H	H
P0493 BstI	H	H	H	H	H	H	H	H	H	H
P23645 MboI	H	H	H	H	H	H	H	H	H	H
P41030 TaqI	H	H	H	H	H	H	H	H	H	H
P45179 BstI	H	H	H	H	H	H	H	H	H	H
B100046 PspI	H	H	H	H	H	H	H	H	H	H
B53066 BsaJI	H	H	H	H	H	H	H	H	H	H
B56691 XbaI	H	H	H	H	H	H	H	H	H	H
B51627 BstZ17	H	H	H	H	H	H	H	J	H	H
B40835 MspI	H	H	H	H	H	H	H	J	H	H
B19839 MspI	H	H	H	H	H	J	H	J	H	H
B2387 BstI	H	H	H	H	J	H	J	H	H	H
C1361 HpaII	H	H	J	J	J	J	J	J	J	J

J: Homozygous for Koshihikari

H: Heterozygous for Koshihikari /MS-FR Koshihikari

Table 1 shows that all the recombinants have an indica-derived Rf-1 chromosomal region between P9493 BstI 5 and 59066 BsaJI. This result showed that recombinant 10 pollens having the chromosomal organization as shown in Fig. 3 have pollen fertility, i.e. the Rf-1 gene is functional in these pollens. This means that a sequence determining the presence of the function of the Rf-1 gene 15 is included in the indica region common to these recombinant pollens, i.e. in a region from the P4497 MboI to B56691 XbaI loci (about 65 kb) as estimated at maximum.

[0161] Reference example 4: Complementation assay for a 15.7 kb fragment from XSG16

15 (1)

(Materials and Methods)

The λ phage clone XSG16 (Figs. 1 and 5) was

partially digested with NotI and electrophoresed on an agarose gel. The separated 15.7 kb fragment (including bases 38538-54123 of SEQ ID NO:1) was purified by QIAEXII (QIAGEN).

5 [0162] On the other hand, an intermediate vector pSB200 having a hygromycin-resistant gene cassette was prepared on the basis of pSB11 (Komari et al., *supra*). Specifically, a nopaline synthase terminator (Tnos) was first fused to a ubiquitin promoter and a ubiquitin intron
10 10 (Pubi-ubiII). A hygromycin-resistant gene (HYG(R)) was inserted between ubiII and Tnos of the resulting Pubi-ubiII-Tnos complex to give an assembly of Pubi-ubiII-HYG(R)-Tnos. This assembly was fused to a HindIII/EcoRI fragment of pSB11 to give pKY205. Linker sites for adding restriction
15 enzyme sites NotI, NspV, EcoRV, KpnI, SacI, EcoRI were inserted into the Hind III site upstream of Pubi of this pKY205 to give pSB200 having a hygromycin-resistant gene cassette.

[0163] After the plasmid vector pSB200 was completely
20 digested with NotI, DNA was recovered by ethanol precipitation. The recovered DNA was dissolved in TE solution and then dephosphorylated by CIAP (TAKARA). The reaction solution was electrophoresed on an agarose gel, and then a vector fragment was purified from the gel using
25 QIAEXII (QIAGEN).

[0164] The two fragments prepared above, i.e. the 15.7 kb fragment from XSG16 and the vector fragment were subjected to a ligation reaction using DNA Ligation Kit

Ver. 1 (TAKARA). After the reaction, DNA was recovered by ethanol precipitation. The recovered DNA was dissolved in pure water (prepared by a Millipore system) and then mixed with *E. coli* DH5 α cells, and the mixture was
5 electroporated. After electroporation, the solution was cultured with shaking in LB medium (37°C, 1 hr) and then plated on an LB plate containing spectinomycin and warmed (37°C, 16 hr). Plasmids were isolated from 24 of the resulting colonies. Their restriction enzyme fragment
10 length patterns and boundary base sequences were analyzed to select desired *E. coli* cells transformed with recombinant plasmids.

[0165] The *E. coli* cells selected above were used for triparental mating with the *Agrobacterium tumefaciens* strain LBA4404/pSB1 (Komari et al., 1996) and the helper *E. coli* strain HB101/pRK2013 (Ditta et al., 1980) according to the method of Ditta et al. (1980). Plasmids were isolated from 6 of the colonies formed on an AB plate containing spectinomycin and their restriction enzyme
20 fragment length patterns were analyzed to select desired *Agrobacterium* cells.

[0166] The *Agrobacterium* cells selected above were used to transform MS Koshihikari (having BT cytoplasm and a nucleus gene substantially identical to Koshihikari) according to the method of Hiei et al. (1994). Necessary immature seeds of MS Koshihikari for transformation can be prepared by pollinating MS Koshihikari with Koshihikari.
25 [0167] Transformed plants were transferred to a

greenhouse under long-day conditions after acclimation. 48 individuals grown to a stage suitable for transplantation were transplanted into 1/5000a Wagner pots (4 individuals/pot), and transferred into a greenhouse under short-day conditions 3-4 weeks after transplantation. About one month after heading, seed fertility was tested on standing plants.

[0168] (Results and Discussion)

Of the 47 transformed individuals, at least 37 10 individuals clearly restored fertility (Fig. 6). This indicates that 15586 bases (bases 38538-54123 of SEQ ID NO:1) derived from rice (IR24) in the 15.7 kb insert fragment include the full-length Rf-1 gene.

[0169] (2) Complementation assay for an internal 15 11.4 kb fragment in XSG16

(Materials and Methods)

The λ phage clone XSG16 was completely digested with AlwNI and BsiWI and then DNA was recovered by ethanol precipitation. The recovered DNA was dissolved in TE 20 solution and then blunted by DNA Blunting Kit (TAKARA). The reaction solution was electrophoresed on an agarose gel to separate a 11.4 kb fragment, which was purified by QIAEXII (QIAGEN).

[0170] The plasmid vector pSB11 (Komari et al. Plant 25 Journal, 1996) was completely digested with SmaI and then DNA was recovered by ethanol precipitation. The recovered DNA was dissolved in TE solution and then dephosphorylated by CIAP (TAKARA). The reaction solution was

electrophoresed on an agarose gel, and then a vector fragment was purified from the gel using QIAEXII (QIAGEN).

[0171] The two fragments prepared above were subjected to a ligation reaction using DNA Ligation Kit Ver. 1 (TAKARA). After the reaction, DNA was recovered by ethanol precipitation. The recovered DNA was dissolved in pure water (prepared by a Millipore system) and then mixed with *E. coli* DH5 α cells, and the mixture was electroporated. After electroporation, the solution was cultured with shaking in LB medium (37°C, 1 hr) and then plated on an LB plate containing spectinomycin and warmed (37 °C, 16 hr). Plasmids were isolated from 14 of the resulting colonies, and their restriction enzyme fragment length patterns and boundary base sequences were analyzed to select desired *E. coli* cells.

[0172] The *E. coli* cells selected above were used for triparental mating with the *Agrobacterium tumefaciens* strain LBA4404/pSB4U (Takakura et al., Japanese Patent Application No. 2001-269982 (WO02/019803 A1)) and the helper *E. coli* strain HB101/pRK2013 (Ditta et al., 1980) according to the method of Ditta et al. (1980). Plasmids were isolated from 12 of the colonies formed on an AB plate containing spectinomycin and their restriction enzyme fragment length patterns were analyzed to select desired *Agrobacterium* cells.

[0173] The *Agrobacterium* cells selected above were used to transform MS Koshihikari (having BT cytoplasm and a nucleus gene substantially identical to Koshihikari)

according to the method of Hiei et al. (1994). Necessary immature seeds of MS Koshihikari for transformation can be prepared by pollinating MS Koshihikari with Koshihikari.

[0174] Transformed plants were transferred to a
5 greenhouse under long-day conditions after acclimation.
120 individuals grown to a stage suitable for
transplantation were transplanted into 1/5000a Wagner pots
(4 individuals/pot), and transferred into a greenhouse
under short-day conditions about one month after
10 transplantation. About one month after heading, one
typical ear was sampled from each plant to evaluate seed
fertility (the percentage of fertile paddies to total
paddies).

[0175] (Results and Discussion)
15 Of the 120 transformed individuals, 59 individuals
showed seed fertility of 10% or more, among which 19
individuals showed seed fertility of 70% or more. This
indicates that the 11.4 kb insert fragment (bases 42357-
53743 of SEQ ID NO:1) contains an essential Rf-1 gene
20 region for expressing a fertility restoring function.

[0176] (3) Complementation assay for an internal 6.8 kb
fragment in XSG16

(Materials and Methods)

The λ phage clone XSG16 was completely digested with
25 HpaI and AlwNI and electrophoresed on an agarose gel. The
separated 6.8 kb fragment was purified by QIAEXII (QIAGEN).

The subsequent procedures including the preparation
of the plasmid vector pSB11 were performed according to

the method in (2) above.

[0177] (Results and Discussion)

Of the 120 transformed individuals, 67 individuals showed seed fertility of 10% or more, among which 26 5 individuals showed seed fertility of 70% or more. This indicates that the 6.8 kb insert fragment (bases 42132-48883 of SEQ ID NO:1) contains an essential Rf-1 gene region for expressing a fertility restoring function.

[0178] Other fragments derived from Asominori shown in 10 Figs. 1 and 5, i.e., XSE1,XSE7,XSF4,XSF4,XSF18,XSF20,XSG22, XSG8,XSH18 and XSX1 were also subjected to complementation assay in a similar manner. However, none of them had a fertility restoring function.

[0179] Reference example 5: Preparation of cDNA library

Firstly, IL216, a line wherein the Rf-1 is introduced into Koshihikari via backcrossing (the genotype, Rf-1/Rf-1), was prepared. The IL216 was grown in a greenhouse by a conventional method, and young panicles 20 were sampled during the growth stage wherein the length between auricles is -5 ~ 5 cm. Total RNA was extracted by the SDS-phenol method (Watanabe, A. and Price, C.A. (1982) Translation of mRNAs for subunits of chloroplast coupling factor 1 in spinach. Proceedings of the National Academy 25 of Sciences of the U.S.A., 79, 6304-6308), and the poly (A)⁺ RNA was purified using QuickPrep mRNA Purification Kit(Amersham Pharmacia Biotech).

[0180] The purified poly (A)⁺ RNA was provided to

prepare a cDNA library by ZAP-cDNA Synthesis Kit (Stratagene). The titer of the prepared library (1ml) was calculated to be 16,000,000 pfu/ml, and was determined to be sufficiently large.

5 [0181] Reference example 6: Screening of the cDNA library

(1) Preparation of the screening primers

PCR was performed by using the following two types of primers:

10 Sense primer

5'-tctcattctccacggctgctc-3' (SEQ ID NO:50)

Antisense primer

5'-acggcgaggacaattcgatcgaaacac-3' (SEQ ID NO:51)

and XSG16, a genomic clone of IR24, as a template. SEQ ID

15 NOS:50 and 51 correspond to the bases 43733-43756 and the bases 44038-44015 of SEQ ID NO:1, respectively.

[0182] After the electrophoresis, the amplification product of about 300 bp was recovered from the agarose gel by QIAEX II Gel Extraction Kit (QIAGEN). The recovered 20 fragment was 32 P-labelled by Rediprime II DNA labelling system (Amersham Pharmacia Biotech) (The fragment is hereunder referred to as "Probe P").

[0183] Further, PCR was performed by using the following two types of primers:

25 Sense primer

5'-agtgtgtggatggtgcatttccg-3' (SEQ ID NO:52)

Antisense primer

5'-ctctacaggatacacggtgtaagg-3' (SEQ ID NO:53)

and XSG16, a genomic clone of IR24, as a template. SEQ ID NOS:52 and 53 correspond to the bases 48306-48329 and the bases 50226-50203 of SEQ ID NO:1, respectively. After the electrophoresis, the amplification product of about 1900
5 bp was recovered from the agarose gel. The recovered fragment was ^{32}P -labeld by the method mentioned above (The fragment is hereunder refers to as "Probe Q").

[0184] (2) Screening of the cDNA library

The cDNA library prepared in Reference example 5 was
10 provided to prepare 70 of agar medium wherein about 15000 plaques appeared. Plaque lift was performed twice for each agar medium, and the plaques were transferred to Hybond-N⁺ (Amersham Pharmacia Biotech). One membrane was used for hybridization with Probe P, and the other
15 membrane was used for hybridization with Probe Q. The whole steps were performed according to the manufacture's instructions.

[0185] Probes were added to a hybridization solution containing 250 mM Na₂HPO₄, 1 mM EDTA and 7% SDS, and
20 hybridization was performed at 65°C for 16 hours. Washing was performed twice with a solution containing 1 X SSC and 0.1% SDS, at 65°C for 15 minutes, and then twice with a solution containing 0.1 X SSC and 0.1% SDS, at 65°C for 15 minutes. After the washing, the membranes were
25 analyzed with FUJIX BAS 1000 (Fuji Photo Films).

[0186] As a result, 8 plaques which showed positive for both Probe P and Probe Q were identified. Therefore, those plaques were isolated, subcloned into pBluescript

according to the instructions of the manufacture (Stratagene). Among 8 clones, the terminal base sequences of 6 clones were identical to that of XSG16. The entire base sequences of the 6 clones were determined, and the 5 results are shown in SEQ ID NOS:43-74 in the sequence listing.

[0187] All of the sequences, SEQ ID NOS:43-74 are presumed to encode a protein having the amino acids 1-791 of SEQ ID NO:49. Specifically, all and each of the 215-10 2587 of SEQ ID NO:43, the bases 213-2585 of SEQ ID NO:44, the bases 218-2590 of SEQ ID NO:45, the bases 208-2580 of SEQ ID NO:46, the bases 149-2521 of SEQ ID NO:47 and the bases 225-2597 of SEQ ID NO:48 encodes a protein having amino acids 1-791 of SEQ ID NO:49. The above base 15 sequences correspond to the bases 43907-46279 of SEQ ID NO:1.

[0188] The amino acid sequence of SEQ ID NO:49 was compared with the presumed amino acid sequence of the corn fertility restorer gene (Rf2), and the N-terminal 7 amino 20 acid residues (Met-Ala-Arg-Arg-Ala-Ala-Ser) in both amino acid sequences were concurred. These 7 amino acid residues are considered to be a portion of a targeting signal to mitochondria (Liu et al., 2001). Based on the above facts, the cDNAs isolated on this occasion are 25 considered to contain the full coding region of the Rf-1 gene. No homology between the amino acid sequences of the rice Rf-1 and the corn Rf2 can be found except for the above region. It is presumed that the mechanisms by which

the gene products of the Rf-1 and the Rf2 can restore fertility after being transferred to mitochondria are distinct from each other.

[0189] In addition, the sequences of cDNAs isolated on 5 this occasion were compared with the genome sequence of IR24 (SEQ ID NO:1), and the structures of exons and introns of the Rf-1 gene were clarified (Fig.7). As a result, it was shown that various transcription products wherein the splicing forms and the poly A addition 10 positions are different, are present in a plant body.

There is no intron in the coding region of the Rf-1 gene.

[0190] Reference example 7: Complementation assay

A complementation assay was performed by using a 4.2 kb fragment containing the promoter region and the 15 presumed translation region of the Rf-1 gene. The 4.2 kb fragment is in a plasmid containing the 6.8 kb genome derive from IR24 which proved to have fertility restorer function in Reference example 4(3).

[0191] Firstly, the plasmid described in Reference 20 example 4(3) was treated with EcoRI, and was subjected to electrophoresis with agarose gel. The 4.2 kb fragment containing the promoter region and the presumed translation region of the Rf-1 (corresponding to the bases 42132-46318 in SEQ ID NO:1) was separated, recovered from 25 the gel using QIAEXII (QIAGEN). The 4.2 kb fragment was subjected to ligation reaction using DNA Ligation Kit Ver.1 (TAKARA) together with pBluescript II SK (-) which has been treated with EcoRI and then with CIAP (TAKARA).

After the reaction, the DNA was recovered by ethanol precipitation.

- [0192] The recovered DNA was dissolved in pure water (prepared by a Millipore system) and then mixed with 5 E. coli DH5 α cells, and the mixture was electroporated. After electroporation, the solution was cultured by shaking in LB medium (37°C, 1 hr) and then plated on an LB plate containing ampicillin and warmed (37°C, 16 hr). Plasmids were isolated from 12 of the resulting colonies, 10 and their restriction enzyme fragment length patterns and boundary base sequences were analyzed to select desired E. coli cells. Then, plasmids isolated from the selected E. coli were treated with BamHI and SalI, and electrophoresed on an agarose gel. The 4.2 kb fragment 15 containing the promoter region and the presumed translation region of Rf-1 was separated, and recovered from the gel using QIAEXII (QIAGEN).
- [0193] On the other hand, TnosJH0072 (an intermediate vector comprising the nos terminator and a cassette of the 20 ampicillin resistant gene) was treated with BamHI and SalI, and electrophoresed on an agarose gel. The 3.0 kb fragment containing the nos terminator and the ampicillin-resistant gene was separated, and was recovered from the gel using QIAEXII (QIAGEN).
- 25 [0194] The 4.2 kb fragment containing the promoter region and the presumed translation region of Rf-1, and the fragment derived from TnosJH0072 were subjected to ligation reaction, and to electroporation by the methods

discussed above. The reactant was spread on LB plates containing ampicillin, and incubated (37°C, 16 hr). Plasmids were isolated from 12 of the resulting colonies, and their restriction enzyme fragment length patterns and 5 boundary base sequences were analyzed to select desired *E. coli* cells.

[0195] Further, plasmids isolated from the selected *E. coli* were treated with SgfI, and electrophoresed on an agarose gel. The 4.2 kb fragment containing the promoter 10 region and the presumed translation region of Rf-1 was separated, and recovered from the gel using QIAEXII (QIAGEN). The 4.2 kb fragment and pSB200Pac (an intermediate vector comprising a cassette of the hygromycin-resistant gene) which has been treated with 15 PacI and then with CIAP (TAKARA) were subjected to ligation reaction, and to electroporation by the methods discussed above. The reactant was spread on LB plates containing spectinomycin, and incubated (37°C, 16 hr). Plasmids were isolated from 16 of the resulting colonies, 20 and their restriction enzyme fragment length patterns and boundary base sequences were analyzed to select desired *E. coli* cells.

[0196] As a result of the above steps, *E. coli* cells were obtained wherein the chimera gene of the fragment 25 containing the promoter region of the Rf-1 and the presumed translation region of the Rf-1 attached with the nos terminator has been inserted within an intermediate vector. The *E. coli* cells were used for triparental

mating with the Agrobacterium tumefaciens strain LBA4404/pSB1 (Komari et al., 1996) and the helper E. coli strain HB101/pRK2013 (Ditta et al., 1980) according to the method of Ditta et al. (1980). Plasmids were isolated
5 from 6 of the colonies formed on an AB plate containing spectinomycin and their restriction enzyme fragment length patterns were analyzed to select desired Agrobacterium cells.

[0197] The Agrobacterium cells selected above were
10 used to transform MS Koshihikari (having BT cytoplasm and a nucleus gene substantially identical to Koshihikari) according to the method of Hiei et al. (1994). Necessary immature seeds of MS Koshihikari for transformation were prepared by pollinating MS Koshihikari with Koshihikari.

15 [0198] Transformed plants were transferred to a greenhouse under long-day conditions after acclimation. 32 individuals grown to a stage suitable for transplantation were transplanted into 1/5000a Wagner pots (4 individuals/pot), and transferred into a greenhouse
20 under short-day conditions 3-4 weeks after transplantation. About one month after heading, seed fertility was tested on standing plants. As a result, 28 individuals among the 32 transformed individuals restored fertility.

[0199] By the above procedures, it has been
25 experimentally demonstrated that the function of the Rf-1 gene can be furnished by expressing the presumed translation region.

[0200] Reference example 8: Isolation of cDNA

In Reference example 6, the cDNA library derived from IL216 young panicles was screened with Probe P and Probe Q. Plaques which are positive for both probes were isolated and analyzed, and 6 cDNA were isolated. In this 5 reference example, similar screening was performed with Probe P and Probe R as mentioned below, and six additional cDNAs were isolated. Details are as follows.

Firstly, PCR was performed by using the following two types of primers:

10 Sense primer

5'-cagttgggttgaacctaatactg-3' (SEQ ID NO:60)

Antisense primer

5'-caactaaaccgttagacgagaaaagc-3' (SEQ ID NO:61)

and a genomic clone of IR24, XSG16 as a template. SEQ ID 15 NOS:60 and 61 correspond to the bases 45522-45545 and the bases 45955-45932 of SEQ ID NO:1, respectively.

[0201] After the electrophoresis, the amplification product of about 430 bp was recovered from the agarose gel by QIAEX II (QIAGEN). The recovered fragment was 20 ^{32}P -labeld by Rediprime II DNA labelling system (Amersham Pharmacia Biotech) (hereinafter referred as "Probe R", Fig. 8).

[0202] The cDNA library derived from IR24 young panicles was provided to prepare 20 of agar medium wherein 25 about 15000 plaques appeared. Plaque lift was performed twice for each agar medium, and the plaques were transferred to Hybond-N⁺ (Amersham Pharmacia Biotech). One membrane was used for hybridization with Probe P of

Reference example 6, and the other membrane was used for hybridization with Probe R. All of the steps were performed according to the manufacture's instructions. As a result, 12 plaques were identified which proved to be
5 positive for both Probe P and Probe R.

[0203] Accordingly, those plaques were isolated, and subcloned into pBluescript according to the instructions of the manufacture (Staratagene). The terminal base sequences of the clones were determined. Among 12 clones,
10 the terminal base sequences of 6 clones were identical to that of XSG16, and thus the entire base sequences of those 6 clones were determined (#7 - #12). The results were shown in SEQ ID NOS:54-85.

[0204] All of the sequences, SEQ ID NOS:54-85 are
15 presumed to encode a protein having the amino acids 1-791 of SEQ ID NO:49. Specifically, all and each of the 229-2601 of SEQ ID NO:54, the bases 175-2547 of SEQ ID NO:55, the bases 227-2599 of SEQ ID NO:56, the bases 220-2592 of SEQ ID NO:57, the bases 174-2546 of SEQ ID NO:58 and the
20 bases 90-2462 of SEQ ID NO:59 encodes a protein having amino acids 1-791 of SEQ ID NO:49. The above base sequences correspond to the bases 43907-46279 of SEQ ID NO:1.

[0205] The sequences of cDNAs isolated on this
25 occasion were compared with the genome sequence of IR24 (SEQ ID NO:1), and the structures of exons and introns were clarified (Fig.8). Among the cDNAs isolated on this occasion, there are three cDNAs which do not have any

exons irrelevant to the presumed translation region, and consist of a single exon (#10 - #12, SEA ID NOS: 57-59).

[0206] Example 1: Selection of Single-Copy Insertion Transformant

5 (Materials and Methods)

The present inventors discovered that, in Reference example 4(1), when a 15.6 kb fragment from the genomic clone XSG16 of IR24 is inserted into MS Koshihikari (which has BT cytoplasm and substantially the same nuclear genes 10 as Koshihikari), seed fertility is restored in primary transformants (T_0 generation).

[0207] Twelve plants were selected from among the primary transformants (T_0 generation) in which fertility had been restored, and total DNA was extracted from green 15 leaves on the selected plants by the SDS-phenol method (Komari et al., 1989). The DNA was completely digested with SacI and submitted to agarose electrophoresis, then transferred to Hybond-N⁺ (Amersham Pharmacia Biotech) in accordance with the manufacturer's manual and furnished to 20 Southern analysis.

[0208] The probe used for Southern analysis was created as follows. First, using two different primers:
5'-attgagggttgaacaatgatggc-3' (SEQ ID NO:62)
(corresponding to bases 49244 to 49267 of SEQ ID NO:1)
25 and
5'-ctctacaggatacacgggttaagg-3' (SEQ ID NO:63)
(corresponding to bases 50226 to 50203 of SEQ ID NO:1),
the PCR was carried out using the above-mentioned genomic

clone XSG16 as the template. Following electrophoresis, an approximately 980 bp amplification product was recovered from the agarose gel with the QIAEX II Gel Extraction Kit (QIAGEN). The recovered fragment was 5 ^{32}P -labeled using the Rediprime II DNA labeling system (Amersham Pharmacia Biotech).

[0209] Hybridization was carried out at 65°C for 16 hours by adding the probe to a hybridization solution containing 250 mM Na₂HPO₄, 1 mM EDTA and 7% SDS. The 10 membrane was washed twice at 65°C for 15 minutes each time with a solution containing 1 x SSC and 0.1% SDS, then washed twice at 65°C for 15 minutes each time with a solution containing 0.1 x SSC and 0.1% SDS. The washed membrane was then analyzed using FUJIX BAS1000 (Fuji Photo 15 Film). Other test methods were carried out while referring to the above-referenced laboratory manual (Sambrook et al., 2001).

[0210] Some of the individuals that were found from the results of SacI digestion to be single-copy 20 transformants were EcoRV digested, then subjected to Southern analysis in the same way as described above.

[0211] (Results and Discussion)
As a result of SacI digestion and Southern analysis on 12 individuals, in addition to the approximately 12 kb 25 band corresponding to the intrinsic rf-1 gene, bands of various sizes were observed. Because the number of bands in the respective individuals is believed to reflect the number of inserted copies in that individual, the seven

individuals in which only one band other than the approximately 12 kb band was observed were treated as candidate single-copy insertion transformants.

[0212] Six of these candidate single-copy
5 transformants were selected and subjected to EcoRV digestion and Southern analysis. As a result, in each individual, one band was observed in addition to an approximately 15 kb band corresponding to the intrinsic rf-1 gene. The foregoing results showed that these six
10 individuals were single-copy insertion transformants.

[0213] Example 2: Selection of Individuals Homozygous for Insertion Gene

(Materials and Method)

Four individuals (16T0-6, 16T0-26, 16T0-34, 16T0-35)
15 of the six that were shown in Example 1 to be single-copy insertion transformants were inbred, and six T₁ individuals for each line were cultivated. The total DNA was extracted by the method described in Example 1 and subjected to EcoRV digestion and Southern analysis.

20 [0214] (Results and Discussion)

One individual homozygous for the inserted gene was selected from each line (16T1-6, 16T1-26, 16T1-34, 16T1-35) by comparing within each line the intensities of the approximately 15 kb band corresponding to the intrinsic
25 rf-1 gene and the band corresponding to the inserted gene. The pollen fertilities of these four individuals were examined by iodine-potassium iodide staining, whereupon all were found to be close to 100%, indicating that the

genotypes of the insertion loci inferred from the Southern analysis results were correct.

[0215] Example 3: Identification of Chromosomal Sites of Inserted Genes

5 (1) Identification of Insertion Site in 16T0-6
(Materials and Method)

The DNA of 16T0-6 used in Example 1 was completely digested with *PstI*, following which amplification of the insertion site was carried out using the LA PCR in vitro 10 Cloning Kit (TAKARA) in accordance with the manufacturer's manual. In the first PCR, Nos F1:

5'-agattgaatcctgttgcggcttgcgatg-3' (SEQ ID NO:64)
was used as the specific primer. The PCR conditions were 2 minutes of treatment at 94°C, followed by 30 cycles, 15 each consisting of 1 minute of thermal denaturation at 94°C, 1 minute of annealing at 58°C and 2 minutes of elongation reaction at 72°C, then 2 minutes of treatment at 72°C at the end.

[0216] The second PCR was carried out with 1 µL of a 20 200-fold dilution of the first PCT solution as the template and using Nos F2:

5'-tcatctatgttaactagatccgatgataaggc-3' (SEQ ID NO:65)
as the specific primer. The PCR conditions were the same as in the first PCR. The second PCR solution was 25 furnished to agarose gel electrophoresis, following which the amplified fragments were recovered from the agarose gel by QIAEX II Gel Extraction Kit (QIAGEN) and the base sequence was analyzed.

[0217] (Results and Discussion)

The second PCR solution was submitted to agarose gel electrophoresis, and approximately 500 bp fragments were recovered. The end base sequences of the recovered
5 fragments were determined, and a BLAST search (Altschul et al., 1990) was performed on the Genbank public database. As a result, the base sequence was found to match the complementary strand sequence of the genomic clone (Accession NO: AP004007) on chromosome 6 on Nihonbare.
10 [0218] Hence, the two following primers were designed for the positions shown in Fig. 9 with respect to the complementary strand sequence of AP004007:

No6 F:

5'-acttcaactagcacccctctcacct-3' (SEQ ID NO:66),

15 and

No6 R:

5'-tctgctggtaaacatggtgtatag-3' (SEQ ID NO:67).

Using these primers, the PCR was carried out with the complete DNAs of Koshihikari and 16T1-6 (an individual
20 homozygous for the inserted gene) described in Example 2 as the templates. The PCR conditions were 2 minutes of treatment at 94°C, followed by 35 cycles, each consisting of 30 seconds of thermal denaturation at 94°C, 30 seconds of annealing at 58°C and 30 seconds of elongation reaction
25 at 72°C, then 2 minutes of treatment at 72°C at the end.

On submitting the PCR solution to agarose gel electrophoresis, the results showed that fragments of the expected size (210 bp) were amplified from the Koshihikari

DNA. However, as expected, the product of interest here was not amplified from 16T1-6.

- [0219] In addition, the PCR was carried out under the foregoing conditions with a primer combination of NosF2 5 and No6R and using the total DNAs of Koshihikari and 16T1-6 as the templates. As a result, fragments of the expected size (234 bp) were amplified from 16T1-6. However, as expected, the product of interest was not amplified from Koshihikari.

10 [0220] The above results show that the insertion site of the inserted gene in 16T0-6 is a site which corresponds with AP004007 on chromosome 6.

[0221] (2) Identification of Insertion Site in 16T0-26
(Materials and Method)

15 The 16T0-26 DNA used in Example 1 was completely digested with PstI, following which the insertion site was amplified by the method described in (1) above, and the base sequence was analyzed. GC Buffer (I) shipped with TaKaRa LA Taq (TAKARA) was used as the PCR buffer.

20 [0222] (Results and Discussion)

The second PCR solution was submitted to agarose gel electrophoresis, and approximately 1700 bp fragments were recovered. The end base sequences of the recovered fragments were determined, and a BLAST search (Altschul et 25 al., 1990) was performed on the Genbank public database. As a result, the sequence was found to match the sequence of the genomic clone (Accession NO: AC026758) of chromosome 10 on Nihonbare.

[0223] The two following primers were thus designed for the positions shown in Fig. 9 on the sequence of AC026758:

No26 F:

5'-cccccccccctctcctct-3' (SEQ ID NO:68),

and

No26 R:

5'-tccccaccaaaggcattcctctcatc-3' (SEQ ID NO:69).

Using these primers, the PCR was carried out with the
10 complete DNAs of Koshihikari and 16T1-26 (an individual
homozygous for the inserted gene) described in Example 2
as the templates. The PCR conditions were 2 minutes of
treatment at 94°C, followed by 35 cycles, each consisting
of 30 seconds of thermal denaturation at 94°C, 30 seconds
15 of annealing at 58°C and 30 seconds of elongation reaction
at 72°C, then 2 minutes of treatment at 72°C at the end.
On submitting the PCR solution to agarose gel
electrophoresis, the results showed that fragments of the
expected size (246 bp) were amplified from the Koshihikari
20 DNA. However, as expected, the product of interest here
was not amplified from 16T1-26.

[0224] In addition, the PCR was carried out under the
foregoing conditions with a primer combination of Nos F2
and No26 R and using the complete DNAs of Koshihikari and
25 16T1-26 as the templates. As a result, fragments of the
expected size (352 bp) were amplified from 16T1-26.
However, as expected, the product of interest was not
amplified from Koshihikari.

[0225] The above results show that the insertion site of the inserted gene in 16T0-26 is a site which corresponds with AC026758 of chromosome 10.

- [0226] (3) Identification of Insertion Site in 16T0-34
5 (Materials and Method)

The 16T0-34 DNA used in Example 1 was completely digested with BamHI, following which the insertion site was amplified by the method described in (1) above, and the base sequence was analyzed.

- 10 (Results and Discussion)

The second PCR solution was submitted to agarose gel electrophoresis, and approximately 1700 bp fragments were recovered. The end base sequences of the recovered fragments were determined, and a BLAST search (Altschul et al., 1990) was performed on the Genbank public database. As a result, as of September 9, 2002, clones having the aforementioned sequences had not been found.

- [0227] (4) Identification of Insertion Site in 16T0-35
(Materials and Method)

20 The 16T0-35 DNA used in Example 1 was completely digested with PstI, following which the insertion site was amplified by the method described in (1) above, and the base sequence was analyzed.

- [0228] (Results and Discussion)

25 The second PCR solution was submitted to agarose gel electrophoresis, and approximately 500 bp fragments were recovered. The end base sequences of the recovered fragments were determined, and a BLAST search (Altschul et

al., 1990) was performed on the Genbank public database. As a result, the sequence was found to match the sequence of the genomic clone (Accession NO: AP004009) on chromosome 7 on Nihonbare.

5 [0229] Hence, the two following primers were designed for the positions shown in Fig. 9 on the sequence of AP004009:

No35 F:

5'-ggcttagggtttggggaaatggcg-3' (SEQ ID NO:70),

10 and

No35 R:

5'-cgtcatcatcttctccaaaacagcc-3' (SEQ ID NO:71).

Using these primers, the PCR was carried out with the complete DNAs of Koshihikari and 16T1-35 (an individual 15 homozygous for the inserted gene) described in Example 2 as the templates. The PCR conditions were 2 minutes of treatment at 94°C, followed by 35 cycles, each consisting of 30 seconds of thermal denaturation at 94°C, 30 seconds of annealing at 58°C and 30 seconds of elongation reaction 20 at 72°C, then 2 minutes of treatment at 72°C at the end. On submitting the PCR solution to agarose gel electrophoresis, the results showed that fragments of the expected size (235 bp) were amplified from the Koshihikari DNA. However, as expected, the product of interest was 25 not amplified from 16T1-35.

[0230] In addition, the PCR was carried out under the foregoing conditions with a primer combination of Nos F2 and No35 R and using the complete DNAs of Koshihikari and

16T1-35 as the templates. As a result, fragments of the expected size (177 bp) were amplified from 16T1-35. However, as expected, the product of interest was not amplified from Koshihikari.

5 [0231] The above results show that the insertion site of the inserted gene in 16T0-35 is a site which corresponds with AP004009 of chromosome 7.

[0232] Example 4: Pollen Fertility Study by Iodine-Potassium Iodide Staining of Cumulative Rf-1 Gene Line
10 (Materials and Methods)

The following plant materials were furnished for testing.

- [0233] 1) MS Koshihikari
2) Koshihikari
15 3) FR Koshihikari (a line established by inserting the Rf-1 gene into Koshihikari by continuous back-crossing)
4) MS Koshihikari x FR Koshihikari
5) self-fertile F₁ plants (16T2-6, 16T2-26, 16T2-34, 16T2-35) of lines 16T1-6, 16T1-26, 16T1-34 and 16T1-35
20 6) MS Koshihikari x 16T1-6, MS Koshihikari x 16T1-26, MS Koshihikari x 16T1-34, MS Koshihikari x 16T1-35
7) FR Koshihikari x 16T1-6, FR Koshihikari x 16T1-26, FR Koshihikari x 16T1-34, FR Koshihikari x 16T1-35
8) 3-loci Rf-1 heterozygote
25 9) 4-loci Rf-1 heterozygote

The 3-loci Rf-1 heterozygote and 4-loci Rf-1 heterozygote of 8) and 9) were created as described below. The 3-loci Rf-1 heterozygote was created by extracting the

DNA from 39 plants obtained by the following cross: (FR Koshihikari × 16T1-6) × (FR Koshihikari × 16T1-35), and estimating the Rf-1 locus genotype, the 16T1-6 insertion locus genotype (chromosome 6) and the 16T1-35 insertion locus genotype (chromosome 7) of each individual as described below using DNA markers. The Rf-1 locus was estimated from the genotype of the S12564 Tsp509I and C1361 MwoI loci in accordance with Komori et al. (2002). In the case of the 16T1-6 insertion locus, if a 234 bp fragment was amplified when the PCR was carried out using the primers Nos F2 and No6 R described in Example 3, the genotype of this locus was regarded as heterozygous. Similarly, in the case of the 16T1-35 insertion locus, if a 177 bp fragment was amplified when the PCR was carried out using the primers Nos F2 and No35 R described in Example 3, the genotype of this locus was regarded as heterozygous. As a result of marker assays, three individuals of the population obtained from this cross were inferred to be heterozygous for the Rf-1 locus, the 16T1-6 insertion locus and the 16T1-35 insertion locus.

[0234] To create a 4-loci Rf-1 heterozygote, the Rf-1 locus genotype, the 16T1-6 insertion locus genotype, the 16T1-35 insertion locus genotype and the 16T1-34 insertion locus genotype were estimated for 62 plants obtained by the following cross: (16T1-34 × 16T1-6) × (FR Koshihikari × 16T1-35). In the case of the 16T1-34 insertion locus, the PCR was carried out using as the primers Nos F2 and No34 R described in Example 3:

5'-cctttatacctccccacttcttatcc-3' (SEQ ID NO:72).

The PCR conditions were 2 minutes of treatment at 94°C, followed by 35 cycles, each consisting of 30 seconds of thermal denaturation at 94°C, 30 seconds of annealing at 5 58°C and 30 seconds of elongation reaction at 72°C, then 2 minutes of treatment at 72°C at the end. The PCR solution was then submitted to agarose gel electrophoresis. If 245 bp fragments were amplified, the genotype of this locus was regarded as heterozygous. As a result of marker assays, 10 five individuals of the population obtained from this cross were inferred to be heterozygous for the Rf-1 locus, the 16T1-6 insertion locus, the 16T1-35 insertion locus and the 16T1-34 insertion locus.

[0235] Four unopened glumous flowers after heading 15 were collected per plant from two plants of each of the above varieties and lines 1) to 9). The anther was removed from each flower, lightly ground in an iodine-potassium iodide solution, then examined under a microscope. Pollens that elicited a deep blue color from 20 the iodine-starch reaction were regarded as fertile pollen, and other pollens were regarded as infertile pollens. At least 200 pollens were examined for each glumous flower.

[0236] (Results and Discussion)
The pollen fertility of each glumous flower was 25 computed. Table 2 shows the results obtained from calculations of the average pollen fertility and standard deviation for eight glumous flowers of each variety and line.

[0237] [Table 2]

Table 2 Result of Pollen Fertility Study by Iodine-Potassium Iodide Staining

Variety · Line	average pollen fertility (%)	standard deviation
MS Koshihikari	0.00	0.00
Koshihikari	97.24	1.12
FR Koshihikari	95.71	1.71
MS Koshihikari × FR Koshihikari	50.17	2.38
16T1-6	95.12	2.79
16T1-26	93.66	1.61
16T1-34	94.08	2.06
16T1-35	95.20	1.20
MS Koshihikari × 16T1-6	51.92	4.03
MS Koshihikari × 16T1-26	53.27	4.37
MS Koshihikari × 16T1-34	49.65	2.81
MS Koshihikari × 16T1-35	51.20	4.19
2-loci Rf-1 hetero (FR Koshihikari × 16T1-6)	74.34	3.78
2-loci Rf-1 hetero (FR Koshihikari × 16T1-26)	91.71	3.04
2-loci Rf-1 hetero (FR Koshihikari × 16T1-34)	70.41	5.18
2-loci Rf-1 hetero (FR Koshihikari × 16T1-35)	75.69	4.72
3-loci Rf-1 hetero	86.28	2.01
4-loci Rf-1 hetero	92.23	1.73

5 The theoretical pollen fertilities of 1) MS
Koshihikari, 2) Koshihikari, 3) FR Koshihikari and 4) MS
Koshihikari × FR Koshihikari are respectively 0%, 100%,
100% and 50%. Pollen fertilities close to these
theoretical values were observed.

10 [0238] The lines in 5) (16T2-6, 16T2-26, 16T2-34, and
16T2-35) exhibited the same degree of pollen fertility as
FR Koshihikari, and the crossed lines in 6) (MS
Koshihikari × 16T1-6, MS Koshihikari × 16T1-26, MS
Koshihikari × 16T1-34, and MS Koshihikari × 16T1-35)
15 exhibited the same degree of pollen fertility as MS
Koshihikari × FR Koshihikari. The implication of these
results is that each of the Rf-1 genes introduced by

genetic engineering techniques functions in the same way as an intrinsic Rf-1 gene.

[0239] The crossed lines in 7) (FR Koshihikari × 16T1-6, and FR Koshihikari × 16T1-35) had pollen fertilities of 5 respectively 74% and 76%. The intrinsic Rf-1 gene carried by FR Koshihikari is located on chromosome 10, whereas the inserted Rf-1 genes carried by 16T1-6 and 16T1-35 are located on chromosomes 6 and 7, respectively, as noted in Example 3. Therefore, in F₁ plants, pollen having both 10 the intrinsic Rf-1 gene and the inserted Rf-1 gene, pollen having only the intrinsic Rf-1 gene, pollen having only the inserted Rf-1 gene, and pollen having neither Rf-1 gene should segregate in a ratio therebetween of 1:1:1:1. Given that the pollen fertilities of these F₁ plants is 15 about 75%, pollen having one or more Rf-1 gene was assumed to be fertile.

[0240] The pollen fertility of FR Koshihikari crossed with 16T1-34 was 70%, which is close to the expected value of 75% when the intrinsic Rf-1 and the inserted Rf-1 are 20 independent. The position of the inserted Rf-1 gene carried by 16T1-34 has not been identified, although these results indicate at the very least that it is a locus which is not strongly linked to the intrinsic Rf-1 locus on chromosome 10.

25 [0241] The pollen fertility of FR Koshihikari crossed with 16T1-26 was 92%. As mentioned in Example 3, the inserted Rf-1 gene carried by 16T1-26 is located inside AC026758 on chromosome 10; AC026758 corresponds to the

RFLP marker locus C797. On the other hand, the intrinsic Rf-1 gene carried by FR Koshihikari is closely linked with the RFLP marker locus S12564 on chromosome 10 (Komori et al., 2002). According to a RFLP linkage map (Harushima et al., 1998), the map distance between C797 and S12564 is about 20 cM. In cases where the frequency of recombination between both markers is about 20%, the theoretical pollen fertility of FR Koshihikari × 16T1-26 is calculated to be about 90%. The observed pollen 10 fertility is close to this theoretical value.

[0242] The 3-loci Rf-1 heterozygote in 8) carries the Rf-1 gene on chromosomes 6, 7 and 10, and so each Rf-1 gene is inherited independently. Therefore, in these individuals, pollen carrying three, two, one and no Rf-1 15 gene are expected to segregate in a ratio of 1:3:3:1. These plants had a pollen fertility of approximately 87.5%, indicating that the pollens having one or more Rf-1 gene were fertile. It was thus assumed that pollen containing three Rf-1 genes develops normally.

[0243] The 16T1-34 insertion locus has not been identified in the 4-loci Rf-1 heterozygote in 9), and so it is unclear whether each Rf-1 gene is inherited independently. However, the pollen fertility observed was very close to the theoretical pollen fertility of 93.75% 25 when it is assumed that each Rf-1 gene is independently inherited and that pollen carrying one or more Rf-1 gene is fertile. It was assumed from this that pollen carrying four Rf-1 genes develops normally.

[0244] Example 5: Pollen Germination Tests on
Cumulative Rf-1 Gene Lines
(Materials and Method)

The following plant materials were furnished for
5 testing.

- [0245] 1) Koshihikari
2) MS Koshihikari × FR Koshihikari
3) FR Koshihikari × 16T1-6, FR Koshihikari × 16T1-35

Four glumous flowers during flowering were selected
10 per plant from two plants for each of the above varieties
and lines. The anther in each flower was snipped off with
tweezers, and placed pollens directly on a pollen
germination medium. In accordance with an earlier report
(Kariya, 1989), an agar medium composed of 1% agar, 20%
15 sucrose and 20 ppm H₃BO₃ was used as the pollen germination
medium. Pollen in which the elongation of pollen tubes
was observable under a microscope after at least 20
minutes had elapsed was regarded as fertile pollen. At
least 200 pollens were examined for each flower.

20 [0246] (Results and Discussion)

The germination rate was computed for each glumous
flower. Table 3 shows the results obtained from
calculations of the average germination rate and standard
deviation for eight glumous flowers of each variety and
25 line.

[0247] [Table 3]

Table 3 Result of Pollen Germination Rate Tests

Variety • Line	average germination rate (%)	standard deviation
Koshihikari	92.85	1.40
MS Koshihikari×FR Koshihikari	38.98	8.16
FR Koshihikari×16T1-6	58.25	9.20
FR Koshihikari×16T1-35	65.91	6.07

The germination rates for Koshihikari and MS

- 5 Koshihikari × FR Koshihikari were respectively 93% and 39%.
The germination rates for FR Koshihikari × 16T1-6 and FR
Koshihikari × 16T1-35 were respectively 58% and 66%; while
not as high as the germination rate for Koshihikari, these
were significantly higher than the germination rate for MS
10 Koshihikari × FR Koshihikari.

[0248] When considered together with the pollen
fertility test results obtained by iodine-potassium iodide
staining, those lines that are heterozygous for the Rf-1
gene at multiple gene loci had an increased proportion of
15 starch-storing pollen (i.e., an increased proportion of
normal development) compared with ordinary hybrids
(heterozygous for Rf-1 at one locus). As a result, the
proportion of pollen that actually germinates also
presumably increases.

- 20 [0249] Example 6: Establishment of a 2-Loci Rf-1
Homozygous Fertile Restorer Line and a 3-Loci Rf-1
Homozygous Fertility Restorer Line

A 2-loci Rf-1 homozygous fertility restorer line was

established as follows. DNA was extracted from 24 F₂ plants obtained by crossing FR Koshihikari with 16T1-6, and the genotypes at the Rf-1 locus and the 16T1-6 insertion locus (chromosome 6) were estimated for each 5 individual. The Rf-1 locus was estimated from the genotypes of the S12564 Tsp509I locus and the C1361 MwoI locus, in accordance with Komori et al. (2002). As for the insertion locus in the 16T1-6 line, if a 210 bp fragment was not amplified when the PCR was carried out 10 using the primers No6 F and No6 R described in Example 3, the genotype of that locus was regarded as homozygous for the introduced gene. Based on the marker assays, one of the plants studied was presumed to be homozygous for the fertility restorer gene at both the Rf-1 locus and the 15 16T1-6 insertion locus.

A 3-loci Rf-1 homozygous fertility restorer line was established as follows. DNA was extracted from 39 plants obtained by carrying out the following cross: (FR Koshihikari × 16T1-6) × (FR Koshihikari × 16T1-35), and 20 the Rf-1 locus, 16T1-6 insertion locus (chromosome 6) and 16T1-35 insertion locus (chromosome 7) in each individual were estimated by means of DNA markers as described below. The Rf-1 locus was estimated from the genotypes of the S12564 Tsp509I locus and the C1361 MwoI locus, in 25 accordance with Komori et al. (2002). In the case of the insertion locus in the 16T1-6 line, if a 234 bp fragment was amplified when the PCR was carried out using the primers No6 F2 and No6 R described in Example 3, the

genotype of that locus was regarded as heterozygous.

Similarly, if a 177 bp fragment was amplified when the PCR was carried out using the primers Nos F2 and No35 R described in Example 3, the genotype of the insertion

5 locus in 16T1-35 was regarded as heterozygous. Based on marker assays, one individual from among the population obtained by this cross was presumed to be homozygous for the Rf-1 gene at the Rf-1 locus and heterozygous for the Rf-1 genes at both the insertion locus in 16T1-6 and the
10 insertion locus in 16T1-35.

[0250] This one individual was self-fertilized and DNA was extracted from 24 offspring in the F₂ generation, following which the 16T1-6 insertion locus and 16T1-35 insertion locus of each offspring were estimated. The
15 genotype of the 16T1-6 insertion locus was regarded as homozygous if a 210 bp fragment was not amplified when the PCR was carried out using the primers No6 F and No6 R as described above. The genotype of the 16T1-35 insertion locus was taken to be homozygous if a 235 bp fragment was
20 not amplified when the PCR was carried out using the primers No35 F and No35 R described in Example 3. Based on marker assays, two individuals from among those studied were presumed to be homozygous for the fertility restorer gene at both the 16T1-6 insertion locus and the 16T1-35
25 insertion locus. Because these individuals were also Rf-1 homozygous at the Rf-1 locus, they were Rf-1 homozygous at a total of three loci.

[0251] Example 7: Cold Hardiness Test

(Materials and Methods)

Koshihikari, the F₁ plants obtained from MS Koshihikari crossed with FR Koshihikari, and F₁ plants obtained from FR Koshihikari crossed with 16T1-35 5 (described in Example 4) were furnished for testing. After being grown by a conventional method up to the transplantation stage, four plants of each variety and line were transplanted to 1/5000 are Wagner pots (1 plant per pot). Following transplantation, the plants were 10 cultivated in an air-conditioned chamber set to 12 hours of lighted conditions (24°C) and 12 hours of darkness (19°C). After ripening, ten panicles from each plant were collected and the seed fertility (proportion of all the caryopsis that have ripened) for each panicle was 15 determined. The average seed fertility for the ten panicles was treated as the seed fertility for that plant.

[0252] (Results and Discussion)

The average seed fertility of four plants was about 95% in Koshihikari, and about 57% in the F₁ generation of 20 MS Koshihikari × FR Koshihikari. Because the F₁ generation of MS Koshihikari × FR Koshihikari had a lower seed fertility than Koshihikari, a comparison between cold-hardy cultivars and lines was thought to be possible under the low-temperature conditions used at this time. 25 Also, the average seed fertility among four F₁ plants obtained by crossing FR Koshihikari with 16T1-35 was about 76%, which, although not as high as for Koshihikari, is nonetheless higher than for the F₁ plants obtained by

crossing MS Koshihikari with FR Koshihikari. In a test conducted on the difference in the population ratio, the difference in seed fertility between F₁ plants obtained by crossing MS Koshihikari with FR Koshihikari (3,276 out of 5,808 caryopses ripened) and F₁ plants obtained by crossing FR Koshihikari with 16T1-35 (4,587 out of 5,900 caryopses ripened) had a significance level of 1%. These results mean that, compared with prior-art hybrids, hybrids which are heterozygous for the Rf-1 gene at a plurality of loci retain a high seed fertility even under low temperature conditions. Hence, it is likely that the cold hardiness of hybrid varieties can be enhanced by using a fertility restorer line which is homozygous for the Rf-1 gene at a plurality of loci.